INFECTIOUS BOVINE RHINOTRACHEITIS IN GREAT BRITAIN IN THE PERIOD 1970 TO 1986

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DECLARATION

All the work reported herein was carried out under my personal direction and control. Except as stated below I personally carried out the animal work, the laboratory studies and the data analyses, with some technical support as noted in the acknowledgements. The laboratory bench work reported in Chapter 4 was done by Heather White assisted by Paula Nixon. The avidin-biotin amplified ELISA studies in Chapter 6 were done in collaboration with George Gitao. The statistical analyses for the IgG ELISA in Chapter 6 were done by Nancy Hebert.

DEDICATION

This thesis is dedicated to the memory of my late father-in-law, Charles Evans (d. 6 April 1988) whose enthusiasm, intellect and wit were a source of constant encouragement in the preparation of this work.

ABSTRACT

Bovid herpesvirus 1 (BHV1) causes a variety of disease syndromes in cattle world-wide. The biology of the virus is reviewed with emphasis on clinical disease, epidemiology and the role of viral latency, the physical, genetic and antigenic make-up of the virus, experimental respiratory disease (infectious bovine rhinotracheitis - IBR), the immune response of cattle and its exploitation in diagnostic tests. An analysis was made of the epidemiology of BHV1 infection in Great Britain, using diagnostic data from Veterinary Investigation Centres and the Central Veterinary Laboratory. There was a marked seasonal incidence, with IBR occurring mainly in winter and fetopathy in summer. There was a dramatic increase in IBR incidence in the late 1970s. Estimates of the prevalence of seropositive cattle showed a rising trend over the period 1970-1986.

Restriction endonuclease fingerprinting of the DNA of BHV1 showed that early British isolates (from both IBR and genital disease) were all of genotype 2 along with the prototype American genital strain. More recent isolates, since 1977, are predominantly of genotype 1 resembling the prototype IBR strains from America. This supports the view that genotype 1 viruses were imported to Britain during the 1970s.

Four British isolates of genotype 1 virus and two of genotype 2 were used in a series of experimental calf inoculations. All six strains produced a febrile upper respiratory and ocular disease. A score system was used to quantify the clinical responses. Statistical analysis indicated that genotype 1 viruses were more virulent than genotype 2, and calves inoculated with genotype 1 excreted significantly more virus. For both genotypes of virus, the calves showed

clinical resistance to subsequent challenge with the opposite genotype. In the primary infections, an early serological IgM response peaked on days 12-14 after inoculation. IgG1 and IgG2 were first detected on days 9 or 10 with a marked rise in IgG1 between days 10 and 15. Secondary rises in IgG antibodies occurred following challenge exposure and also after reactivation of latent virus by the administration of dexamethasone.

Comparisons were made of the diagnostic tests for BHV1 infection. Antigen-detection methods were specific and sensitive although they could not equal the sensitivity of virus isolation. Direct immunofluorescent labelling of cells extracted from the nasal mucus or the use of reverse passive haemagglutination to detect soluble antigen in secretions, were the simplest techniques which offered high sensitivity. A number of enzyme immunossays for cytological labelling or the detection of soluble antigen were evaluated but their technical complexity led to increased opportunities for test error. In contrast, enzyme linked immunosorbent assay proved a flexible and reliable tool for serological diagnosis. The parameters of an IgG assay were determined so as to enable its use in a variety of diagnostic applications. Preliminary studies suggested that an IgM capture assay using monoclonal antibodies was of potential diagnostic value as an indicator of recent infection.

LIST OF ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
AI	artificial insemination
AVI	anti-viral immunoglobulin
BAB	bridged avidin biotin
BHV1	bovid herpesvirus 1
BHV2	bovid herpesvirus 2
BHV4	bovid herpesvirus 4
BHV6	bovid herpesvirus 6
BSA	bovine serum albumen
BVDV	bovine virus diarrhoea virus
CC	cell culture
CHV1	cervid herpesvirus 1
CL	confidence limits
CMI	cell mediated immunity
CVL	Central Veterinary Laboratory
DAB	di-amino benzidine tetra-hydrochloride
DAFS	Department of Agriculture and Fisheries for Scotland
EDTA	ethylene di-amine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EYL	cell culture medium based on Earle's salts
FA	fluorescent antibody
FITC	fluorescein isothiocyanate
gp	glycoprotein
HRPO	horseradish peroxidase
IBR	infectious bovine rhinotracheitis
IPB	infectious pustular balanoposthitis
IPV	infectious pustular vulvo-vaginitis
Kbp	kilo-base pairs
KD	kilodaltons
LAB	labelled avidin biotin
LSD	least significant difference
MAFF	Ministry of Agriculture, Fisheries and Food
MD	megadaltons
MDBK	Madin Darby bovine kidney cell line
MEM	Eagle's minimal essential medium
NS	not significant
OD	optical density
OPD	o-phenylene di-amine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBSL	PBS with lactalbumen hydrolysate (swab elution medium)
PBST	PBS with 0.05% tween 20
PEG	polyethylene glycol
PX	immunoperoxidase
RE	restriction endonuclease
RPHA	reverse passive haemagglutination
SDS	sodium dodecyl sulphate
SHV1	suid herpsvirus 1
SS	simple sandwich
TCID50	50% cell culture infectious units
VI	veterinary investigation
VIDAII	Veterinary Investigation Diagnosis Analysis II
VN	virus neturalization
WOAD	Welsh Office Agriculture Department

Chapter 1

THE BIOLOGY OF BOVID HERPESVIRUS 1: A LITERATURE REVIEW.

INTRODUCTION

This review will focus on those diseases of cattle in which bovid herpesvirus type 1 (BHV1) has been assigned a major causal role. It will extend into the epidemiology, diagnosis and control of the diseases, as well as introducing the molecular biology of the virus, in so far as it relates to the disease epidemiology.

The subject was comprehensively reviewed by Gibbs and Rweyemamu (1977). Clinical aspects were also reviewed by Kahrs (1977). For this reason, the earlier literature will be quoted only selectively in order to present a cohesive view of the virus and its associated diseases in a historical context. Developments in the decade since 1977 will be reviewed in more detail, notably in the areas of immunoassays and the molecular biology of the virus. An introductory review of the latter was given by Ludwig (1982), and summarized by Ludwig and Gregersen (1986).

CLINICAL DISEASES

1. Historical

The earliest reports are found in Germany, in the nineteenth and early twentieth centuries, (summarized by Witte, 1933) of a condition called *Bläschenausschlag* or coital exanthema. Zwick and Gminder (1913, quoted by Ludwig, 1982) proposed a viral aetiology and this was proved by Reisinger & Reimann (1928) who showed its transmissibility to cattle by filtrates of infected secretions.

Kendrick and others (1958) isolated a virus from genital lesions in

cows in the USA. They recognized the similarity of the disease to *Bläschenausschlag* and referred to it as infectious pustular vulvovaginitis (IPV).

Meanwhile, also in the USA, an upper respiratory disease of cattle in beef feedlots had been recognized in Colorado since 1950 (Miller, 1955). Its incidence was increasing and it subsequently emerged as a syndrome of considerable economic importance (Schroeder & Moys, 1954; McKercher and others, 1954). In 1955 the term infectious bovine rhinotracheitis (IBR) was first used (McKercher and others, 1955) and the acronym has since come into widespread use to describe both the disease and its causal virus, which was first isolated by Madin and others (1956). Subsequently it was shown, by cross-neutralization tests in vitro and cross-protection tests in cattle, that the viruses isolated from *Bläschenausschlag*, IPV and IBR were serologically indistinguishable (Gillespie and others, 1959; McKercher, 1963).

2. Infectious Bovine Rhinotracheitis

Darbyshire and others (1962) first isolated the virus in Britain from an outbreak among store cattle in Oxfordshire in 1961 of a disease of high morbidity characterised by conjunctivitis and lachrymation, together with rhinitis and nasal discharge in some animals. The virus was shown to be indistinguishable from the American IBR isolate, and it has since been recognised as the UK prototype strain, designated "Oxford". Dawson and others (1962) described the outbreak in more detail. There was an intense conjunctivitis, uni- or bilateral, with oedema, punctate haemorrhages and profuse lachrymation. Keratitis and photophobia were not seen in uncomplicated cases. Congestion of the nasal mucous membranes was particularly obvious in the

white-faced Hereford cross-bred animals. It was accompanied by a serous to mucopurulent nasal discharge, with areas of necrosis of the nasal mucous membranes. Several animals had slight diarrhoea and occasional animals a moist cough. Uneventful recovery occurred after 10 days' clinical illness. These clinical features resembled those of IBR, described in the USA (Schroeder & Moys, 1954; McKercher and others, 1954; Miller, 1955) although appeared to be relatively mild.

A number of outbreaks have been reported in America (Abinanti and Plumer, 1961; Hughes and others, 1964), Ireland (Timoney and O'Connor, 1971) and Britain (Veterinary Investigation Service, personal communications) where conjunctivitis and ocular discharge were the only presenting signs.

When outbreaks of a severe type of IBR were first reported in Britain (Wiseman and others, 1978), clinical features similar to those described above were present, together with pyrexia, dullness, frequent coughing and tachypnoea. The clinical features of 15 incidents of severe IBR in northern Britain were described by Wiseman and others (1980). There was a reduction in appetite, dullness, pyrexia, soft coughing, ocular discharge, initially serous and later purulent nasal discharge, and nasal mucosal congestion followed by the development of diphtheritic plaques. Drooling of saliva was frequently observed but was not associated with oral lesions. Halitosis, respiratory stertor and slight to moderate tachypnoea were recorded, but clinical pneumonia was rarely a feature even though extensive lung lesions were found in all cases examined pathologically (Allan and others, 1980). The morbidity varied from 10% upwards but was over 90% in ten of the 15 incidents, while the mortality ranged from zero

in five of the incidents up to 7% in the most severe outbreak. Impaired production was noted in that beef cattle showed a reduced rate of weight gain and dairy cows' milk yield fell. Abortions occurred in four of the 15 outbreaks.

Allan and others (1980) described in detail the pathology of 25 natural cases of severe IBR, of which eight had died and the remainder were slaughtered during the acute clinical phase. There were severe acute necrotising rhinitis, pharyngitis, and laryngotracheobronchitis in most of the cases, with pseudomembrane formation and obstruction of the larynx with necrotic debris. The ciliated epithelium of the airways was extensively denuded. Neutrophil infiltration of the epithelia was extensive; macrophages and lymphocytes were also numerous. The pneumonia was of an exudative type, often accompanied by emphysema or pleurisy. Congestion and oedema of the conjunctiva were present in 80 per cent of the cases. Renal infarction was noted in 48 per cent of the cases. No pathological lesions were found in the brains. Although BHV1 was isolated from the 14 cases examined microbiologically, a variety of other pathogens were also found including *Pasteurella spp* (4 cases) and *Mycoplasma bovis* (7 cases). The pathology described must therefore be regarded as complicated BHV1 infection. It has been claimed by Wiseman and others (1984) that the severe form of IBR recognised in northern Britain in the late 1970s was "identical in almost every respect to the disease described originally in North America" in the 1950s.

3. Infectious pustular vulvovaginitis and balanoposthitis.

Although, as mentioned above, IPV had long been recognised in female cattle, it was only with the development of artificial insemination

techniques that attention focussed on the role of the bull as a virus carrier and the potential risk of dissemination of the virus through semen from infected bulls. Bouters and others (1960) isolated BHV1 from bulls with ulcerative infectious pustular balanoposthitis (IPB) and showed that it could induce IPV in heifers. The genital disease was often accompanied by nasal ulceration and discharge from which virus could also be isolated. In Germany (Knoblauch, 1962; Straub and Mackle, 1965) and subsequently in Britain (Huck and others, 1971) outbreaks of IPB were described in bull studs. Saxegaard (1966) described the isolation of BHV1 from 23 out of 24 bulls at an artificial insemination centre, 20 of them being clinically normal. Spradbrow (1968) isolated the virus from frozen semen from a healthy bull. It has also been shown that infected bulls can shed BHV1 intermittently for prolonged periods in excess of one year (Snowdon, 1965; Bitsch, 1973). These findings led to the establishment of BHV1 control programmes for artificial insemination centres in a number of countries, including Britain (Lucas, 1986).

Bulls with IPB may or may not have a visible discharge (mucopurulent or even haemorrhagic) from the preputial orifice (Huck and others, 1971). Lesions were only evident after extrusion of the penis and varied from papules through haemorrhages and pustules to frank ulceration. Ocular and/or nasal signs were also present in some bulls. Concurrent IBR, IPV and IPB in a dairy herd were also described by Collings and others (1972). The principal genital signs in the females were tail swishing, frequent micturition, oedema and erythema of the vulvo-vaginal mucosa, and purulent vaginal discharge which could persist for weeks. Focal pustular lesions were found in

the vaginal mucosa during the acute stage of the disease. The disease in many animals was mild (Kendrick and others, 1958) and was not always apparent on external examination. The effects on appetite and milk yield were minimal. A more severe form of BHV1 genital infection has been described in Africa (Kaminjolo and others, 1975), associated with epididymitis and vaginitis.

4. Infertility and Abortion

The role of BHV1 in infertility remains uncertain. Witte (1933) challenged earlier views that IPV and infertility were associated, and the controversy continues in the literature to the present. The early literature was reviewed by Saxegaard (1970) who found some evidence for impaired semen quality in acutely infected bulls. In females he concluded that natural mating with infected bulls did not affect the conception rate, but artificial insemination with infected semen may cause endometritis and hence prevent conception. This view was supported by the experimental work of Parsonson and Snowdon (1975) who found that natural mating of susceptible females with preputially infected bulls produced lesions of IPV, but did not significantly affect the conception rate or the number of services per conception compared with the same bulls before they were infected. In contrast, the inoculation of BHV1 into the uterus at the time of first insemination led to a greatly reduced pregnancy rate and increased services per conception (4.5) compared with non-infected controls (1.7 services per conception). In this case the infected group suffered endometritis, salpingitis and vaginitis, and had a high proportion of shortened oestrous cycles (<18 days). Detailed studies of the pathogenesis of this phenomenon (Miller and Van der

Maaten, 1984 and 1985; Van der Maaten and Miller, 1985; Van der Maaten and others, 1985) have shown that the adrenal glands and the corpus luteum of dioestrus are highly susceptible to infection with BHV1 (including attenuated vaccine strains). Necrotic lesions were produced in these organs, and plasma progesterone concentrations were abnormally low, following intramuscular, intravenous or intra-uterine inoculation of the virus at, or 1 day after, oestrus. Ovarian infections did not develop following nasal aerosol inoculation. This was thought to be because such animals did not develop viraemia.

Bowen and others (1985) showed that recently hatched embryos were highly susceptible to *in vitro* exposure to BHV1. Viral replication led to embryonic death. Bielanski and others (1987) showed a similar susceptibility in early embryos from which the zona pellucida had been artificially removed. Miller and Van der Maaten (1986) extended these observations to *in vivo* studies by inoculating BHV1 intravenously into pregnant heifers 7, 14, 21 or 28 days after mating. Necrotic corpora lutea were found in the 7 and 14 day inoculations, and necrotic follicles in the 21 and 28 day heifers, all the ovarian lesions being associated with viral antigen. Of the two animals inoculated at seven days after mating, one had a normal conceptus and the other a dead, virus-infected embryo.

It may be concluded therefore that infertility associated with BHV1 could be caused by:

(a) Reluctance of bulls to mate due to painful IPB.

(b) Impaired semen quality in infected bulls.

(c) Abnormal hormonal events in females due to infection of the corpora lutea and adrenal glands.

(d) Failure of implantation due to viral endometritis.

(e) Early embryonic death due to viral infection of the embryo.

Whilst infertility is considered as a sequel to viral infection at mating or insemination, abortion (which has long been associated with BHV1 - Owen and others, 1964) is regarded as a sporadic sequel to the respiratory disease (IBR). The received dogma on the pathogenesis of abortion by BHV1 is taken from Kendrick (1973 and 1980). He claims that the virus spreads by the haematogenous route, during the acute phase of IBR, to reach the placenta where a chronic infection is established. Some time, possibly many weeks later, an unknown trigger mechanism allows the virus to cross the placenta and reach the fetus. BHV1 viraemia during acute infection is not readily demonstrated and usually requires cocultivation of blood leukocytes with susceptible cell monolayers. Peripheral blood monocytes support replication of the virus and are probably the vehicle of haematogenous spread (Nyaga and McKercher, 1980). Once infected, fetuses undergo a fulminating infection and die within a few days with extensive focal necrotic lesions throughout the body, notably in the liver, kidney and spleen. Experiments in our laboratory (S. Edwards and C. Richardson, unpublished work) have confirmed that 100 day bovine fetuses are dead by 5 days after direct intrafetal inoculation of BHV1. Nevertheless most natural abortions due to BHV1 occur during the second half of gestation. Kendrick and Osburn (1973) showed that fetuses of between 3 and 8 months gestation could respond serologically to inactivated BHV1 antigen. It is therefore presumed that the absence of BHV1 antibody in aborted fetuses (Lucas and others, 1986) is because fetal death supervenes before seroconversion can occur. The incidence of

BHV1 abortion appears to vary widely. Kirkbride and others (1973) in the USA found it was the commonest diagnosed cause of abortion (16% of cases) and epizootics in the absence of respiratory disease have been reported in Germany (Straub and others, 1982) and Hungary (Tanyi and others, 1983). Although sporadic cases have occurred in Britain in the past decade (Wiseman and others, 1980; Stubbings and Cameron, 1981; Nettleton and others, 1981), usually in association with IBR outbreaks, BHV1 abortion does not appear to have given any great cause for concern here (Lucas and others 1986).

5. Acute Systemic Disease of Newborn Calves.

Some time in the last month of gestation the fetus undergoes a change which enables it to survive an acute BHV1 infection and mount an immune response (Kendrick, 1973). There is evidence that such a late gestationally infected fetus could be born alive as a virus carrier (Pospisil and others, 1979). The finding that calves born to seropositive dams could be reared free of BHV1 infection (Barth and others, 1983; Bradley, 1985) suggests that this late gestational *in utero* transmission is an uncommon event. The change over to relative resistance to BHV1 appears to be variable in onset; there are a number of reports of an acute fatal systemic disease of newborn calves, in which the lesions resemble those of BHV1 infected aborted fetuses (Reed and others, 1973; Higgins and Edwards, 1986). Mechor and others (1987) reproduced the disease experimentally by aerosol exposure of two day old calves to BHV1, and also demonstrated clinical protection by prior feeding of seropositive colostrum. Others have found typical, but severe, signs of the respiratory form of IBR in young calves (Thiry and others, 1984), or an ulcerative alimentary

tract disease (Ross and others, 1983). The latter disease (in the absence of bovine virus diarrhoea infection - which it resembles) has also been recorded in older cattle (Rogers and others, 1978). Msolla and others (1983a) reported evidence from experimental infections in calves that the susceptibility to IBR continued to decline for some months after birth, and suggested that the ability to resist infection was related to the maturation of the cell-mediated immune system.

6. Other Diseases.

Alimentary tract disease has been mentioned above. BHV1 has also been isolated from udder lesions (Guy and others, 1984) and from cows' milk (Probst and others, 1985). The other major disease syndrome associated with BHV1 is encephalitis (French, 1962; Carrillo, 1983). This has not been reported in Britain, although histological evidence of a viral encephalitis was reported by Higgins and Edwards (1986).

7. Other Species

Viruses antigenically related to BHV1 have been isolated from pigs, buffalo, goats, sheep and deer, and antibodies to BHV1 have been found in these and other ruminant species (reviewed by Gibbs and Rweyemamu, 1977; also Doyle and Heuschele, 1983; Rosadio and others, 1984; Inglis and others, 1983; Brake and Studdert, 1985). Recent advances in virus typing must cast doubt on whether serological reactions, or even virus isolations, reported earlier were associated with BHV1 itself or with related strains such as bovid herpesvirus 6 (BHV6) from goats (Saito and others, 1974; Engels and others, 1983; Hasler and Engels, 1986) or cervid herpesvirus 1 (CHV1) (Reid and

others, 1986) from deer.

Experimentally, rabbits are readily infected (Lupton and others, 1980) and can be used as a laboratory model; mice are relatively refractory although have been infected with some difficulty (Geder and others, 1981).

8. Mixed Infections

Although BHV1 is an established primary pathogen, several disease outbreaks have been described where bovine virus diarrhoea virus (BVDV) has also been isolated (Gratzek and others, 1966; Eugster and others, 1975; Greig and others, 1981). Clinically the outbreaks presented as complex diseases with both respiratory and alimentary signs. Experimental studies indicated that infection with BVDV (a known immunosuppressive agent) allows BHV1, given by aerosol, to invade a much wider range of tissues than it does when given alone (Potgieter and others, 1984). BHV1 has also been isolated from a rinderpest case (Hassan and El Tom, 1985). In interpreting such reports, the possibility should be considered of consequential reactivation of a latent BHV1 infection due to immunosuppression by the primary infectious agent, although an attempt to demonstrate such an effect experimentally with BVDV was unsuccessful (Edwards & Roeder, 1983). BHV1 itself has immunosuppressive effects (Briggs and others, 1988) which may predispose to secondary infection with other agents.

BHV1 infections of the eye do not induce a keratitis *per se,* but can predispose to *Moraxella bovis* infection leading to infectious keratoconjunctivitis (Pugh and others, 1970). Another interaction with

bacteria, *Pasteurella haemolytica,* has been extensively exploited by workers in Canada as a reproducible model of calf pneumonia (Yates and others, 1983a; Jericho, 1983). The combined infection with BHV1 and, four days later, *P. haemolytica* produced more severe lesions than either agent alone. The response was dose dependent for both the virus (Yates and others, 1983b) and the bacteria (Yates and others, 1983c).

EPIDEMIOLOGY AND THE ROLE OF VIRAL LATENCY

Evidence of BHV1 infections in cattle has been reported from all the inhabited continents and the virus is considered to have a worldwide distribution (Odend'hal, 1983). Specific national eradication programmes are in operation in Switzerland (Riggenbach, 1985) and Denmark (Danish Veterinary Service, 1985). The seropositive prevalence in cattle varies widely between and within countries, for example 12% in Scotland (Msolla and others, 1981), 40% in Italy (Allegri and others, 1985), 57% in New Zealand (Durham and Sillars, 1986), 63% in Morocco (Mahin and others, 1985) and 7 to 69% in Egypt (Hafez and Frey, 1973). The widespread practice of vaccination against BHV1 in North America (Kahrs, 1977) confounds any attempts to estimate the natural seroprevalence there.

It has been proposed that BHV1 originated as a venereally transmitted genital infection of cattle (McKercher, 1959) in which the virus and host had evolved to an epidemiologically stable relationship. Increasing intensivism of cattle husbandry gave opportunity for other means of transmission, and there is evidence for non-venereal spread in some outbreaks of IPV/IPB (Kendrick and others, 1958; Straub and Bohm, 1963; Huck and others, 1971) even though venereal transmission

by natural or artificial insemination remains an important factor in the genital disease (Collings and others, 1972; Loretu and others, 1974). Hyne and Johnston (1964) and Collings and others (1972) speculated on the possible role of flies in BHV1 transmission in the outbreaks they described. Conclusive evidence to implicate arthopods in the biology of BHV1 was produced by Taylor and others (1982) who isolated the virus from soft ticks *(Ornithodoros coriaceus)* in the Western USA, although any role for the ticks in transmission to cattle remains to be demonstrated.

McKercher (1963) suggested that the virus must have entered the USA with infected cattle before 1930 (when cattle importation from Europe ceased) and became established there in the form of a low prevalence, relatively mild genital infection (Kendrick and others, 1958) similar to the European situation. IBR was considered to be a new disease, not recognised before about 1950 (Miller, 1955). McKercher (1959) concluded that it arose as a result of BHV1 evolving to exploit the opportunity for rapid transmission by the respiratory route, in the intensive conditions of the American feedlot system. Outbreaks of IBR typically occurred following the introduction of cattle to a herd. There was moderately slow spread over a period of weeks until most cattle in the herd had been affected. This evolutionary theory was supported by the work of Gillespie and others (1959) who showed that whilst IBR and IPV virus isolates were serologically identical, and both could infect cattle by either vaginal or nasal routes, the amount of virus shed in the nasal secretions was 100-fold more for the IBR isolate than for the IPV strain.

In the 1970s, reports of IBR in Europe in a new, severe form (Van Bekkum and Straver, 1975; Wellemans, 1975; Martel and others, 1976; Wiseman and others, 1978) bore close resemblance to the 1950s accounts from the USA (McKercher and others, 1955; Wiseman and others, 1984). Circumstantial evidence suggested that this wave of new disease was associated initially with herds which had imported Holstein cattle from North America (Dannacher and Fedida, 1978; Msolla and others, 1981). This would support the view that certain European countries, including mainland Britain, had imported cattle infected with the IBR strain of BHV1 which evolved in the USA in the 1950s (Ludwig and Gregersen, 1986).

There is general agreement in the literature that outbreaks of IBR in epidemic form usually occur following the introduction of new cattle to a herd (McKercher, 1959; Wiseman and others, 1980). Once established in a herd the virus may circulate intermittently, with long periods (2 to 3 years) of quiescence (Van Nieuwstadt and Verhoeff, 1983). In such herds resurgence of infection may be associated with clinical disease in young non-immune cattle; nevertheless most BHV1 infections pass unnoticed. All the available evidence points to carrier cattle as the main reservoir of BHV1 infection, and BHV1 survives in nature by an interplay of short cycle and latent infections (Gibbs and Rweyemamu, 1977). While other species may become infected there is no evidence that they have any role in perpetuation of the virus (Ackermann and others, 1986; Hasler and Engels, 1986).

The ability to establish latent (ie persistent but non-productive) infections is a characteristic of the family *Herpesviridae* (Rapp and Jerkofsky, 1973). Latent infection of cattle by BHV1 was implied by

observations of intermittent virus shedding over long periods (Snowdon, 1965). Sheffy and Davies (1972) were the first of many authors to report the consistent recrudescence of latent BHV1 in seropositive cattle from 3 days after the administration of corticosteroid hormones. Other events which can induce reactivation of latent BHV1 include other viral infections (parainfluenza type 3 - Mensik and others, 1976), parasitic infections (*Dictyocaulus viviparus* - Msolla and others, 1983b), 3-methylindole toxicity (Espinasse and others, 1983), parturition (Thiry and others, 1985a) and transport of the animals (Thiry and others, 1985b and 1987). The pathogenesis of BHV1 latency and its role in the epidemiology of the virus have been reviewed by Pastoret and others (1982 and 1984). A theoretical model for BHV1 latency, based on binary logic, has been proposed by Pastoret and others (1986).

Davies and Duncan (1974) showed that the local sensory nerves (trigeminal for the respiratory tract and sacral for the genital tract) were closely related to the persistence and latency of BHV1 in experimentally infected calves. Further studies have implicated the trigeminal and sacral nerve ganglia as the major site of viral latency, using the techniques of ganglion explant culture (Homan and Easterday, 1980) or in situ DNA hybridization (Ackermann and others, 1982; Homan and Easterday, 1983; Ackermann and Wyler, 1984). The latter technique has shown the latent viral DNA to be located in the nuclei of the ganglionic neurons. At the time of recrudescence, ganglionitis could be demonstrated histopathologically, associated with demonstrable viral antigen (Narita and others, 1978a, 1979a and 1981). During the quiescent (latent) phase viral antigen could not

be detected but the presence of viral RNA in the neuronal nuclei (Rock and others, 1986 and 1987) suggested that the BHV1 genome was transcriptionally active at a restricted level during latent infection. The nature and sites of herpesvirus latency and the mechanisms involved in reactivation and recrudescence remain uncertain despite considerable reserch efforts (Wildy and others, 1982). The ganglia may not be the only site of BHV1 latency: Pastoret and others (1979) have speculated on the possible role of latently infected nasal epithelial cells, particularly in relationship to reactivation by dexamethasone. This hormone may exert a direct pharmacological effect on the latently infected cells and this, as well the drug's role as an immunosuppressant (Babiuk and Rouse, 1979), may be involved in the reactivation of latent virus (Pastoret and others, 1982).

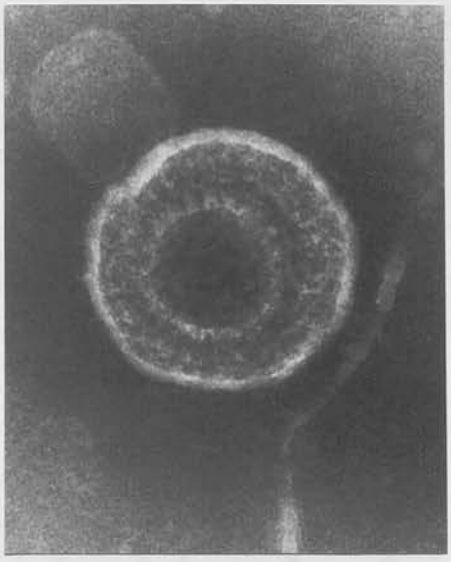
Whatever the mechanisms of latency and reactivation, the epidemiologically important facts for BHV1 are that: all cattle are considered latently infected after recovery from acute infection (Pastoret and others, 1984); the virus is readily reactivated and repeated reactivations can be induced in a single animal (Narita and others, 1979a; Pastoret and others, 1979); reactivated virus is shed at high infectious titre in the nasal and/or genital secretions, whatever the original site of infection (Davies and Duncan, 1974); vaccination either before or after exposure to wild-type virus neither prevents nor abolishes latent infection (Straub, 1979; Nettleton and Sharp, 1980; Zuffa and others, 1985) while live vaccine strains, including temperature sensitive mutants, can themselves establish latent infections and be reactivated artificially or spontaneously (Pastoret and others, 1980a; Gregersen and Wagner, 1985); and the biological

characteristics of recrudescent BHV1 isolates do not appear to differ from the original infecting strain (Castrucci and others, 1984; Rodriguez and others, 1984).

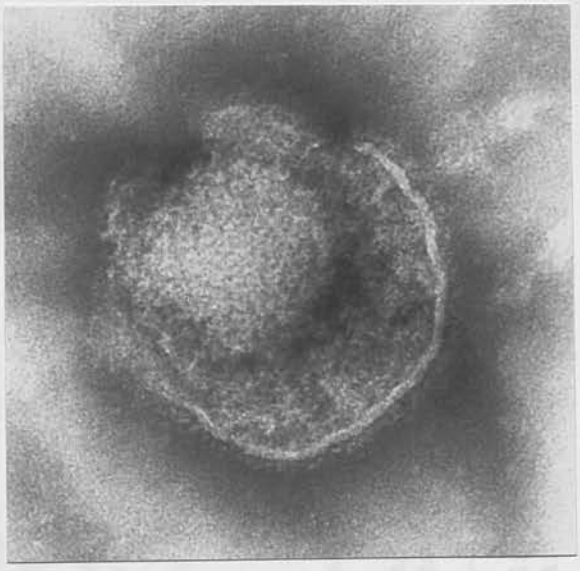
THE VIRUS

The IBR isolate of Madin and others (1956) was shown to be a herpesvirus, with properties similar to herpes simplex virus, by Armstrong and others (1961). It has a double stranded DNA genome, an icosahedral capsid with 162 capsomeres and a surrounding envelope of approximately 200 nm diameter (Fig. 1.1). The virus is sensitive to ether, acid and heat. Its buoyant density is in the range 1.22-1.25 g/cm^3. Haemagglutination of mouse erythrocytes has been demonstrated (Trépanier and others, 1985). The physical and chemical properties of the virion were reviewed by Gibbs and Rweyemamu (1977).

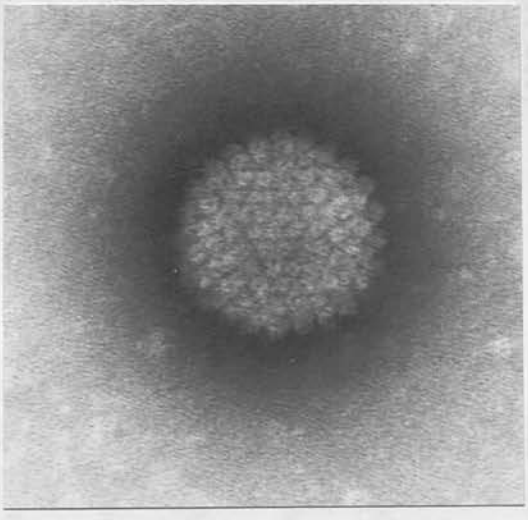
There is no consensus in the literature on the most appropriate nomenclature for this virus. The vernacular names IBR virus or IBR-IPV virus continue to be commonly used. The Herpesvirus Study Group (chairman Roizman, 1973) of the International Committee for the Nomenclature of Viruses classified herpesviruses according to the taxonomic family of the vertebrate host, with arabic numerals to distinguish different viruses of the same host group. Thus IBR virus became bovid herpesvirus 1. This system has been perpetuated by Ludwig (1982 and 1984) and is used in the present account, although the Herpesvirus Study Group had by then decided (Roizman and others, 1981) that it was useful to distinguish sheep and goat herpesviruses from those of cattle, and that classification should therefore be by host subfamily. BHV1 would then be bovine herpesvirus 1. The differences between the two systems are summarised in table 1.1.



(a) magnification x 190 000



(b) magnification x 230 000



(c) magnification x 230 000

Fig. 1.1 Electron micrographs of negatively stained bovid herpesvirus 1 (strain ED1) showing (a) intact virion (b) virion with ruptured envelope, revealing detail of nucleocapsid (c) naked nucleocapsid.

TABLE 1.1 The Classification of Ruminant Herpesviruses (based on Ludwig, 1984, plus other sources quoted in text).

Virus Name (by host family)	Alternative Name (by host subfamily)	Vernacular Name(s) and associated diseases	Natural Host	Herpesvirus Subfamily
Bovid Herpesvirus 1	Bovine herpesvirus 1	IBR, IPV	Cattle	Alpha
Bovid Herpesvirus 2	Bovine herpesvirus 2	Bovine herpes mammillitis, Allerton virus	Cattle	Aplha
Bovid Herpesvirus 3	Alcelaphine herpesvirus 1 and 2	Bovine malignant catarrhal fever	Wildebeest Hartebeest	Gamma (?)
Bovid Herpesvirus 4	Bovine Herpesvirus 3 Bovine Herpesvirus 4	Movar 33/63, DN 599, bovine cytomegalovirus	Cattle	Beta (?)
Bovid Herpesvirus 5	Caprine Herpesvirus 1	Ovine pulmonary adenomatosis associated virus	Sheep	(?)
Bovid Herpesvirus 6	Caprine Herpesvirus 2 (also called Caprine Herpesvirus 1 by some authors)	IBR-like disease of goats	Goats	Alpha
Cervid Herpesvirus 1	-	IBR-like disease of deer	Red Deer	Alpha
Suid Herpesvirus 1	-	Aujeszky's Disease/pseudorabies (fatal in cattle)	Pigs	Alpha

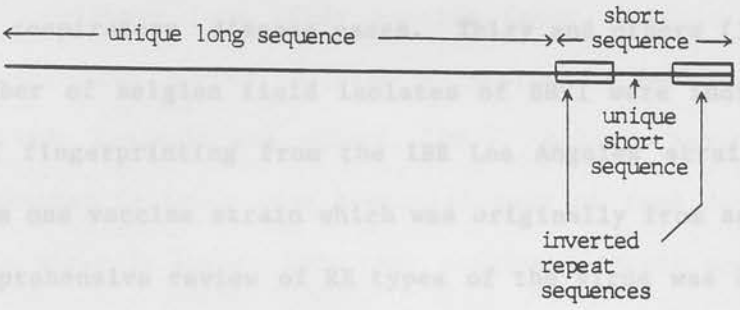
Note: BHV1, BHV6 and Cervid Herpesvirus 1, show antigenic relatedness but are distinguishable by restriction endonuclease cleavage sites on their DNA.

They are of little consequence when considering BHV1, but have caused considerable confusion over the nomenclature of malignant catarrhal fever virus, the Movar 33/63 group and the caprine/ovine herpesviruses (Bartha and others, 1987). BHV1 has the characteristics of the subfamily *Alphaherpesvirinae*, within the family *Herpesviridae*, namely a short replicative cycle, a lytic type of cytopathic effect in culture, and latency in neural ganglia of the host species (Roizman and others, 1981).

The DNA of BHV1 is double stranded with guanine plus cytosine content of 71% (Russell and Crawford, 1964). These authors estimated the molecular weight as 54 mega daltons (MD), but more recent estimates are in the range 84-88 MD (Mayfield and others, 1983; Seal and others, 1985). Skare and others (1975) used BHV1 (the IPV strain - K22) as a heterologous control for their studies of herpes simplex virus, and in doing so published the first restriction endonuclease (RE) fingerprint of BHV1 DNA using the enzyme Eco RI. Restriction endonucleases (type II) are bacterial enzymes which cleave double stranded DNA molecules only at specific base sequences, typically from 4 to 6 base pairs. Their function is to protect the bacteria against foreign DNA, the bacterial DNA itself being protected from digestion by specific methylation at the equivalent base sequences. The enzymes are extensively exploited in genetic manipulation procedures (Malcolm, 1981). They have also been used with a variety of DNA viruses, including herpesviruses (Skare and others, 1975; Paul and others, 1982) to fingerprint strains and isolates by digesting the viral DNA at the specific cleavage loci, then separating the resultant fragments on the basis of molecular weight, by electrophoresis in agarose gels. The technique allows a much more precise definition of virus strains

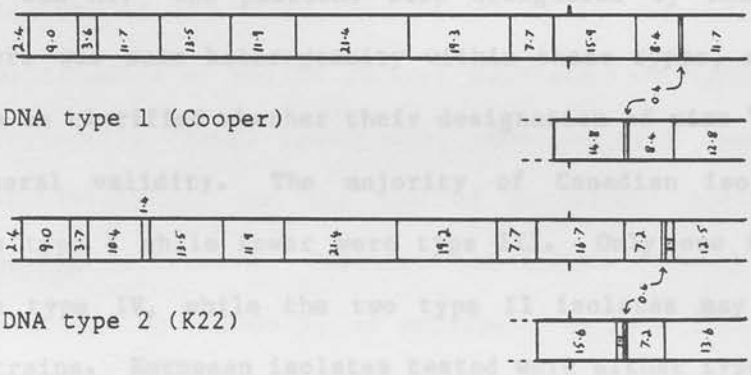
than is possible by antigenic analysis, and it thus allows very detailed epidemiological tracing operations to be carried out (Buchman and others, 1979; Allen and others, 1983).

The BHV1 DNA molecule (Fig. 1.2) consists of a long unique sequence of base pairs, joined to a short sequence composed of two 11.4-12.2 kilobase repeat sequences inverted with respect to each other and flanking a 10.7 kilobase short unique sequence (Farley and others, 1981; Mayfield and others, 1983). Because of the inverted repeat sequences the short sequence can have two orientations with respect to the long sequence, giving two isomeric forms of the DNA. Recent work has shown some heterogeneity in a tandem repeat sequence at the 5' terminal of the unique long sequence (Hammerschmidt and others, 1986). Hammerschmidt and others (1988) sequenced the BHV1 DNA at the genomic termini and at the junction between short and long segments. They proposed functional relationships between the genomic structures, the cleavage of replicative circularized forms of the DNA, and the production of the two isomeric forms of the molecule. Mayfield and others (1983) identified the cleavage loci on the molecule for four restriction enzymes (Hind III, EcoR I, Hpa I, and Bam HI) with the American IBR strain "Cooper" and compared the results with those for the IPV strain "K22". This indicated an overall difference in base sequence of about 5% between the two strains. This was confirmed by Seal and others (1985) who used DNA liquid hybridization to establish that there was at least 95% sequence homology between different isolates of BHV1 despite detectable differences in their RE fingerprints. A preliminary study by Engels and others (1981) indicated that there were at least two patterns of RE



Hind III cleavage sites

(showing the short region in both orientations, with fragment sizes in kilobases)



Hpa I cleavage sites

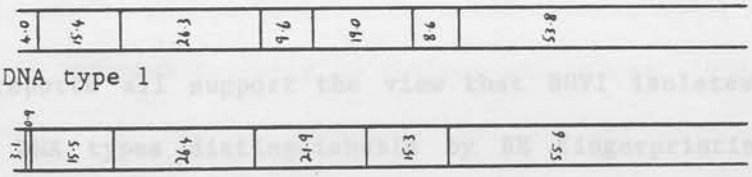


Fig 1.2 Structure of the BHV1 DNA molecule (after Mayfield and others, 1983)

fingerprint which they equated with IBR and IPV isolates. A close inspection of the sources of their isolates does not support such a distinction in that some of their so-called IPV strains were isolated from respiratory disease cases. Thiry and others (1983) found that a number of Belgian field isolates of BHV1 were indistinguishable by RE fingerprinting from the IBR Los Angeles strain, but did differ from one vaccine strain which was originally from an IPV case. A more comprehensive review of RE types of the virus was carried out in Canada (Misra and others, 1983). They identified four major RE types by combining the results of digestions with Eco RI, Hind III, Hpa I and Bam HI. The patterns were designated by Roman numbers I-IV. There was some heterogeneity within these types, although it remains to be clarified whether their designation of nine "sub-types" is of general validity. The majority of Canadian isolates were classed as type I while fewer were type III. Only one isolate was classed as type IV, while the two type II isolates may have been vaccine strains. European isolates tested were either type I or III, including the Oxford and Aberdeen strains from the UK which were both type III.

European reports all support the view that BHV1 isolates fall into two major DNA types distinguishable by RE fingerprinting (Ludwig, 1984; Nettleton and others, 1984; Pauli and others, 1984; Metzler and others, 1985). A simpler, more pragmatic, classification has been proposed (Metzler and others, 1985) by which they are designated RE type 1 (equivalent to type I of Misra and others (1983), "Cooper-like" or "IBR-like") and type 2 (types II and III of Misra and others (1983), "K22-like" or "IPV-like"). This scheme will be followed in the present account.

Recent reports have identified a type 3 virus from cases of encephalitis in cattle in Australia and Argentina (Metzler and others, 1986; Engels and others, 1986), and have also shown that antigenically related herpesviruses from other species (goat, buffalo, deer) are quite distinct from bovine isolates by RE fingerprinting (Brake and Studdert, 1985; Ronsholt and others, 1987). All the bovine respiratory and genital isolates from Australia were of type 2, suggesting that type 1 virus is absent from that continent (Brake and Studdert, 1985).

Our knowledge of the protein structure of the BHV1 virion has expanded considerably in the 1980s, and is likely to continue to do so, with the application of sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) techniques, combined with immunoprecipitation, immunoblotting, and the use of monoclonal antibodies for precise epitope definition. Pastoret and others (1980b) identified 21 viral proteins by SDS-PAGE, of molecular weights from 31 to 275 kilodaltons (KD), and further showed that ten of them were glycosylated. Subsequent work has identified more structural polypeptides - initially 25 (11 glycosylated) (Misra and others, 1981; Metzler and Wyler, 1983) and more recently 33 (Bolton and others, 1983; Chang and others, 1986). Of the 11 glycoproteins, 10 are expressed on the surface envelope of the virion (Chang and others, 1986). A further 15 non-structural viral proteins have been found in BHV1 infected cells (Misra and others, 1981) but are absent from purified virions. Virus-specific thymidine kinase activity has been shown in infected cells (Weinmaster and others, 1982; Kit and Qavi, 1983) and thymidine kinase negative mutants were avirulent for calves (Kit and others, 1985). The location in the viral genome of the thymidine kinase gene

has been mapped (Bello and others, 1987) as has the putative DNA polymerase gene, which hybridized specifically to the equivalent herpes simplex gene (Owen and Field, 1988).

Various authors do not agree on the precise molecular weights of the BHV1 proteins but, this apart, a reasonably consistent picture is now emerging of the envelope glycoproteins and their role in antibody mediated virus neutralization. The seven major immunogenic envelope glycoproteins (gp) comprise three antigenic sets:

(a) *180KD: a dimer of a 90-97 KD gp (neutralizing and haemagglutinating activity and attachment to cells).

(b) 150KD: a dimer of a 74-77 KD gp (the major site for neutralizing activity).

(c) 130KD: a heterodimer of a 33KD and a 74KD gp (involved in complement dependent neutralization).

Ludwig and Letchworth (1987) termed these gIII, gIV and gI glycoproteins respectively and showed that gI and gIV were early (beta) proteins wheras gIII was expressed later (gamma) in the cycle of infection, after the synthesis of virion DNA.

The BHV1 viral proteins have also been used to investigate relationships of the virus with other herpesviruses and to compare isolates

*Molecular weights all approximate; data collated and summarized from Gregersen and others, 1983 and 1985; Collins and others, 1984; Pauli and others, 1984; Van Drunen Littel Van Den Hurk and others, 1984 and 1985; Van Drunen Littel Van Den Hurk and Babiuk, 1985 and 1986a; Lum and Reed, 1986; Marshall and others, 1986; Okazaki and others, 1986; Trépanier and others, 1986; Trudel and others, 1987; Okazaki and others, 1987.

within the BHV1 group. A number of authors have demonstrated serological cross-reactions, by ELISA and neutralization tests, between BHV1 and suid herpesvirus 1 (SHV1) in naturally or experimentally infected or immunised cattle as well as in experimental rabbits (Aguilar Setién and others, 1979b, 1979c and 1980b; Zuffa and others, 1983, Bush and Pritchett, 1986). In one study (Zuffa and others, 1986) cattle vaccinated against BHV1 appeared to be protected against virulent SHV1 challenge. Reciprocal DNA-DNA hybridization indicated 8% sequence homology between the two viruses, with the homologous sequences distributed throughout the genomes (Bush and Pritchett, 1985) located in three regions of the long unique region and one in the inverted repeat segment (Burger and others, 1984). Western blots indicated a two-way cross reaction between the viruses, involving nine SHV1 proteins, and three BHV1 proteins of 224, 125-130, and 58-63KD (strong reactions) plus two weaker reactions at 87 and 55KD (Bush and Pritchett, 1986). Levings and others (1984), using two dimensional immunoelectrophoresis, identified two common antigens in four herpesviruses of cattle: BHV1, BHV2, BHV4 and SHV1. A much closer relationship exists between BHV1 and BHV6 (Gregersen and others, 1983) but even here the cross reactivity (by immunoprecipitation with monoclonal antibodies) was much weaker than within the BHV1 group (DNA types 1, 2 and 3) (Friedli and Metzler, 1986 and 1987).

Comparative studies of BHV1 isolates were made by Bagust (1972) using two isolates from IPV, one from IBR and one from meningitis, all of Australian origin. Recent DNA typing (Brake and Studdert, 1985) suggests that Bagust was in fact working with DNA types 2 (IPV and IBR isolates) and 3 (meningitis). The four strains showed no

differences in thermostability at 40°C or in buoyant density of the virions, but the meningitis strain produced smaller plaques in cell culture. Reciprocal cross neutralization tests showed that the meningitis strain was antigenically different from though still closely related to the other three. In experimental calves all four strains produced rhinitis, conjunctivitis and vaginitis when applied to the respective mucosae, but in addition the meningitis strain alone produced meningo-encephalitis after intranasal or intravaginal inoculation. House (1972) studied the growth characteristics *in vitro* of 12 American isolates. Although there were minor differences between strains, these could not be related to the tissue from which the virus was originally isolated. In Britain, Wojciechowski (1974) showed that the Oxford (IBR) and Carmarthen (IPB) isolates did not differ in their plaque size and morphology, and only slightly differed in the kinetics of reciprocal cross neutralization. It is now known that both strains were of DNA type 2 (see below, Chapter 4).

Pastoret and others (1980b) proposed that IBR and IPV isolates could be distinguished using the SDS-PAGE technique on the basis of minor variations in the molecular weight of three proteins at approximately 99, 68 and 66 KD. Because of the overlap in antigenicity and pathogenicity between BHV1 isolates, it is only following initial DNA typing by the RE fingerprint that meaningful comparisons can be made between the viral proteins of different strains. Misra and others (1983) found only minor variations in the molecular weights of polypeptides among their nine RE "subtypes" of BHV1. Gregersen and others (1985) also found only minor differences in polypeptide patterns and molecular weights between DNA types 1 and 2, while no antigenic differences were demonstrable either in reciprocal cross

neutralization or immunoprecipitation using hyperimmune rabbit sera. That the genetic differences between DNA types 1 and 2 are reflected in the antigenic makeup of the viruses, is now being revealed by the application of monoclonal antibodies (Metzler and others, 1985). This technique has also confirmed that DNA type 3 isolates are clearly distinct from types 1 and 2 (Metzler and others, 1986; Friedli and Metzler, 1986 and 1987).

EXPERIMENTAL INFECTIOUS BOVINE RHINOTRACHEITIS

McKercher and others (1963) infected calves intranasally with the Los Angeles strain of BHV1. They concluded that the primary site of viral multiplication was the nasal epithelium whence it spread by the lachrymal ducts to the eye where it established conjunctivitis, and in blood leukocytes to other organs, although they were unable to detect viraemia.

Markson and Darbyshire (1966) gave a detailed account of the clinical and pathological responses of 15 calves inoculated with the Oxford strain of BHV1 by the intranasal and conjunctival routes. A relatively mild disease was induced, whose main features were pyrexia and intense conjunctivitis with lachrymation. Virus was recovered from the upper and lower respiratory tracts, the conjuctiva, and, in one calf killed 5 days after inoculation, the mediastinal lymph node and spleen. Three calves showed gross pneumonitis. Non-purulent submucosal rhinitis, tracheitis, bronchitis and cholecystitis were regularly found, together with a slight non-purulent encephalitis in four calves. Encephalitis (in the absence of clinical nervous signs) was also described, following intranasal inoculation of BHV1, by Edington and others (1972) and Narita and others (1979b). Narita and

others (1978b) also produced a histological encephalitis by intra-conjunctival inoculation of the virus. They showed that the virus spread to the nasal epithelium and induced a mild rhinitis, in addition to the intense conjunctivitis produced by this route of inoculation, in agreement with the earlier work of Abinanti and Plumer (1961) and Hughes and others (1964).

Schroyer and Easterday (1968) reproduced IBR experimentally by exposure of calves to aerosols of BHV1. Clinical signs included fever, dyspnoea, depression, anorexia and serous or purulent nasal discharge. Virus could be isolated up to 11 days after infection. They also showed contact spread to susceptible calves with clinical signs apparent 5 days after contact, compared with 2 days incubation for the aerosolised calves. Pathologically, they noted extensive loss of the ciliated epithelium of the respiratory tract, together with infiltration of the submucosa by neutrophils, eosinophils and mononuclear cells. Jericho and Darcel (1978) also used aerosol exposure of calves to BHV1 in order to evaluate the effect of ambient temperature and humidity on the disease response. They hoped for wide exposure of the whole respiratory tract to the infection, and a quantitatively consistent exposure of individual calves although they were not satisfied on the latter point. The calves developed a febrile response but the only clinical respiratory sign was coughing. No correlation could be made between the clinico-pathological response and climatic conditions. The pathological lesions included alveolitis and bronchiolitis with epithelial necrosis and complete occlusion of some bronchiolar lumens. In the major airways (bronchi, trachea, nose) there was severe epithelial damage including denudation of cilia or even complete loss of columnar cells. This extensive loss

of cilia in the BHV1 infected respiratory tract was confirmed in scanning electron microscope studied by Allan and Msolla (1980). The consequent damage to pulmonary clearance may be a factor in the synergistic interaction between BHV1 and certain bacteria in inducing respiratory disease (Yates and others, 1983a).

The severity of experimental IBR appears to be related to the age of cattle infected. Webster and Manktelow (1959) produced severe IBR in week-old calves by combined intranasal, conjunctival and intratracheal inoculation. There were necrotic rhinitis, bubbling tracheal sounds, cough, conjunctivitis and fever. At necropsy there was tracheitis with pseudomembrane formation. The same inoculum in yearlings and adults produced only mild disease. Similar age differences were reported by Msolla and others (1983a) who found that 2-week-old or 5-week-old Ayrshires were more severely affected than 6 or 18-month-old Friesians. In contrast, Narita and others (1982) found more severe disease in adult cows than in young calves. A definitive experiment on age susceptibility, in which other variables are carefully controlled, has yet to be reported.

It was established 20-25 years ago that different BHV1 isolates showed little tissue tropism, and would readily infect either the respiratory or the genital tract, whatever the tissue of origin (Gillespie and others, 1959; McKercher, 1963; Maré, 1964; House, 1972; Bagust, 1972). Furthermore, virus was shed in both respiratory and genital secretions following either nasal or vaginal inoculation (House, 1972). It was reported that the respiratory disease induced by intranasal inoculation of IPV isolates was milder than that produced by IBR isolates (Maré, 1964; Gibbs and Rweyemamu, 1977) but such reports are somewhat confounded by variations in the response to

different IBR isolates (Markson and Darbyshire, 1966). The absence of definitive strain markers, until the recent advent of RE fingerprinting, makes interpretation of such comparative pathogenicity studies difficult. As recently as 1983 a comparison of two British IBR isolates (Oxford and Strichen) and one from America (Colorado) (Msolla and others, 1983c) relied solely on descriptive clinical and pathological reports to compare the pathogenicity for calves. They hinted at preliminary studies which indicated that the Strichen strain was of RE type 1 and different from the Oxford strain. The calves infected with Strichen (isolated from severe IBR in Scotland - Wiseman and others, 1978) developed signs and pathological lesions of severe IBR, similar to, but slightly more severe than, those infected with Colorado. The Oxford strain produced a mild clinical response with minimal pathological lesions.

Despite the considerable literature on experimental BHV1 infections there is little evidence of any attempt to quantify the disease response. Descriptive clinical assessment and pathology have been widely used. These methods have a valuable place, but are of limited application when attempting to compare strains of the same virus which produce very similar diseases, differing only in severity. Thomas and others (1977) proposed a quantitative method of evaluating respiratory disease in calves. They used it in studies of parainfluenza type 3 and of combined respiratory syncytial virus and bovine viral diarrhoea virus infections in calves. The clinical response was measured by a score which combined the findings of a daily clinical examination, including rectal temperature, with weekly measurements of haematological parameters. The experiments were based on principles established for human common cold research (Tyrell, 1963)

in which experimental subjects were allocated randomly to treatments, including controls; neither patient nor clinical examiner were aware of treatment allocations; and an individual baseline was established by daily examinations for four days before inoculation. The double blind nature of the clinical evaluations greatly facilitated statistical analysis of the findings. Earlier attempts to quantify respiratory disease in calves included the "index of illness" used by Frank and Marshall (1971) for experimental parainfluenza type 3 infections. This was based on presence/absence of fever, leukopenia and any clinical signs. It indicated the duration but not the severity of disease. McKercher and others (1972) used analysis of variance techniques to evaluate the degree of protection afforded to calves in vaccination and challenge experiments with parainfluenza type 3 virus. They statistically analysed only inherently quantitative data - rectal temperature, antibody titres, and virus shedding. Elazhary and others (1980) used a slightly modified version of the system of Thomas and others (1977) for experimental respiratory syncytial virus infection in calves. Verhoeff and Van Nieuwstadt (1984) extended the application to naturally occurring respiratory disease on farms, in order to evaluate the efficacy of a respiratory syncytial virus vaccine.

Roney and others (1985) were the first to use numerical scores to evaluate clinical responses to BHV1, in this case to assess the effect of interferon on the disease. They produced a daily "lesion score" for each calf by which progression of the disease could be displayed graphically. Statistically, they compared mean lesion scores over the monitoring period between treatment groups. Rectal temperatures were analysed separately and not included in the lesion scores.

THE BOVINE IMMUNE RESPONSE TO BHV1

The immunology of BHV1 infections in cattle has been the subject of considerable study. It has been reviewed by Aguilar-Setién and others (1980a) and Splitter and others (1985), as well as, in the broader context of vertebrate immunity to herpesviruses in general, Rouse and Babiuk (1978), Babiuk and Rouse (1979) and Rouse and Horohov (1984). In acute infections, cellular immunity, mediated by T lymphocytes or the interaction of antibody and Fc receptor bearing cells, is important in recovery from infection, whereas humoral immunity plays a major role in preventing re-infection. Antibody is also involved in maintaining the state of latent infection, while cellular mechanisms come into play in controlling recrudescent infections after reactivation.

Most studies have concentrated on single components of the immune response and it is not yet possible to give a holistic account of the phenomenon. Davies and Carmichael (1973) and Rouse and Babiuk (1974a) demonstrated a virus-specific cell mediated immune (CMI) response by antigen stimulation *in vitro* of blood lymphocytes from BHV1-infected cattle. The response was first detected 5 days after intranasal infection, peaked at days 7 to 10, and declined again by day 19. Similar findings were reported by Tsvetkov and others (1987) who noted that the sudden drop in virus excretion at about day 8 after infection corresponded with the peak of the lymphocyte blastogenic response and preceded the serum antibody response. An alternative assay of lymphocyte activity was viral-plaque inhibition in cell culture (Rouse and Babiuk, 1975). Subsequently it was shown that the CMI activity was found in the T lymphocyte fraction of peripheral blood (Rouse and Babiuk, 1974b; Splitter and Eskra, 1986).

There was considerable variation in T cell responsiveness to BHV1 antigen between individual animals, but this could not be related to breed of cattle (Miller-Edge and Splitter, 1986). Specific cytotoxic activity against BHV1-infected targets was demonstrated in non-adherent lymphocytes from immune cattle by Rouse and Babiuk (1977). The activity was not restricted by the histocompatibility antigens of the targets, and, although they proposed T lymphocytes as the effectors, it has since been suggested they were observing natural killer cell activity (Rouse and Horohov, 1984). BHV1 specific, genetically unrestricted cytotoxicity was also shown in an uncharacterised adherent cell fraction by Campos and Rossi (1985b). The effect was independent of antibody and was not due to natural killer cells. It peaked at 7 days after intranasal inoculation. Classic, histocompatibility antigen restricted, cytotoxicity directed against BHV1 infected target cells has now been demonstrated with T lymphocytes from the blood of immune cattle (Campos and Rossi, 1986).

Antibody-dependent cell-mediated cytotoxicity (ADCC) has received considerable attention and appears to be an important component of the bovine immune response to BHV1. K cells (lymphocyte effectors of ADCC in man) could not be detected in cattle (Campos and Rossi, 1985a). The major bovine effector of ADCC against BHV1 infected targets is the polymorphonuclear leukocyte (Wardley and others, 1976; Thorne and others, 1984; Campos and Rossi, 1985a) although mononuclear phagocytic cells also show ADCC activity (Rouse and others, 1976). The antibodies involved in ADCC were first detected from 4 to 7 days after intranasal infection (Hanton and others, 1986). They reached a plateau after 2 weeks and persisted for at least 9 weeks. The ADCC reaction was enhanced by complement (Rouse and others, 1977;

Hanton and others, 1986), although Grewal and Rouse (1980) also demonstrated antibody independent cytotoxicity (ie non-specific immunity) against BHV1 infected targets by neutrophils in the presence of complement.

Other aspects of CMI which have been described include leukocyte migration inhibition (Aguilar-Setién and others, 1979a) and delayed type hypersensitivity measured by skin test (Aguilar-Setién and others, 1978 and 1983). The latter has been of particular interest for its diagnostic potential.

As well as systemic immunity, local responses in the respiratory tract have a significant effect on the pathogenesis of, and recovery from, IBR (Le Jan and Asso, 1981). The cellular component of the local response includes the alveolar macrophages and infiltrating neutrophils. Alveolar macrophages can support the replication (poorly) of BHV1 *in vitro* and *in vivo* (Forman and others, 1982a and b). Macrophages infected in culture showed reduced ability to mediate ADCC, reduced Fc receptors and phagocytosis, and an initial increase followed by a marked reduction in complement receptors (Forman and Babiuk, 1982). *In vivo* studies on calves given BHV1 by aerosol, and macrophages recovered by lavage, showed complex effects and in general suggested a stimulation of the macrophages (Ohmann and Babiuk, 1986). It was suggested that subsets of the macrophage population may respond in different ways. Bouffard and others (1982) showed a specific cellular response in broncho-alveolar washings from infected calves. Cells recovered at 18 days after infection, but not pre-infection or at 30 days after infection, inhibited the replication of BHV1 in tracheal organ cultures. McGuire and Babiuk (1984) demonstrated impaired neutrophil function in BHV1 infected calves and considered

this was a major factor in the pathogenesis of the BHV1 - *Pasteurella haemolytica* model of calf pneumonia. Neutrophil infiltration into the lung, in response to the bacterial infection, was delayed by prior exposure to the virus and allowed the bacteria to establish more readily.

Humoral factors, both specific (antibody) and non-specific (interferon), play an important role in local immunity. In young calves, maternal antibody of colostral origin (IgG1 class) was found in the nasal secretions, and gave protection against virulent virus challenge (Pospisil and others, 1983). The local active antibody response to BHV1 respiratory infections includes IgA and IgG components and shows virus neutralizing activity (Gerber and others, 1978; Le Jan and Asso, 1981; Rodak and others, 1983). Local immunity has been of particular interest in the development of intranasally administered live virus vaccines (Todd and others, 1971; Todd, 1975; Savan and others, 1979; Zygraich, 1980). BHV1 is a potent interferon inducer (Todd and others, 1972; Fulton and Pearson, 1980; Ahl and Straub, 1985) although BHV1 itself is less susceptible to the anti-viral effects of interferon than are some other respiratory viruses such as parainfluenza 3 and respiratory syncytial viruses (Fulton and others, 1984 and 1986).

The humoral component of the systemic immune response has received considerable attention, not only for the facility with which the assays can be carried out but also for its importance as a diagnostic aid. More attention has perhaps been given to the methodology of antibody assays than to the functional significance of antibodies in immunity to BHV1. Earlier work on BHV1 serology was included in the review by Gibbs and Rweyemamu (1977). A virus neutralizing serum

antibody response is a consistent finding in animals recovering from acute primary infection, and may persist for years (Chow, 1972). The earliest antibodies, after primary infection, were detected at day 7 or 8 and were mainly of the IgM class (Rodak and others, 1983; Guy and Potgieter, 1985a). IgM showed neutralizing activity only in the presence of complement (Rossi and Kiesel, 1974; Potgieter, 1975). IgM peaked at 14 days then decreased rapidly, being replaced by IgG1 (which neutralized in the absence of complement) by a month after primary infection (Rossi and Kiesel, 1976; Guy and Potgieter, 1985a). Secondary exposure to BHV1 in immune cattle produced an anamnestic response which may or may not include an IgM component (Rossi and Kiesel, 1976) but was predominantly IgG2 (Guy and Potgieter, 1985a). The form of the secondary response may have depended on the nature of the antigenic exposure. Reactivation of latent BHV1 infections by the administration of dexamethasone initiated a secondary immune response, again with an IgM component but predominantly IgG1 and IgG2 (Guy and Potgieter, 1985b; Viso and others, 1986). Bitsch (1973) showed that in cattle undergoing repeated cycles of reactivation and latency, the antibody titre tended to rise at each recrudescence but with smaller and smaller increments as the plateau antibody titre increased and the reactivation phases became less frequent.

Collins and others (1985b) and Van Drunen Littel Van Den Hurk and Babiuk (1986b) investigated the reactivity of bovine immune sera with the individual viral polypeptides. It is difficult to compare the results of the two studies because of discrepancies in protein molecular weight estimations, but both seem to agree that a major component of the antibody response is directed against an antigenically related cluster of glycoproteins of 102, 96, 69 and 55 KD

(Collins and others, 1985b) or 130, 74 and 55 KD (Van Drunen Littel Van Den Hurk and Babiuk, 1986b). These studies were extended by Babiuk and others (1987) who immunised cattle with individual BHV1 glycoproteins and showed neutralizing and ADCC antibody responses, as well as protection against the BHV1-*P.haemolytica* model of disease.

DIAGNOSIS OF IBR

The methods available for the diagnosis of IBR were described by Nettleton (1986). In its severe epidemic respiratory form, IBR is a distinctive clinical syndrome characterised by upper respiratory and ocular signs combined with pyrexia. Many practising veterinarians have relied solely on clinical examination to provide a diagnosis. Other forms of BHV1 infection are less easily recognized, and even IBR can be confused with other respiratory and ocular diseases, particularly in less severe cases. Laboratory confirmation of BHV1 infection is therefore frequently indicated and sought, with a demand for rapid tests which can enable early intervention to control an outbreak.

Detection of the virus

A wide range of bovine cell cultures is permissive for BHV1 growth. The virus produces a clearly recognizable cytopathic effect within a few days of inoculation, and confirmatory tests for the virus identity can then be carried out (Schipper and Chow, 1968). Primary bovine cell cultures are the most sensitive system for growth of BHV1 (Gibbs and Rweyemamu, 1977) and isolation in cell culture remains the most sensitive method for detection of the virus even though it involves a delay in reporting of possibly up to 14 days (Nettleton, 1986). The use of culture for diagnosis is facilitated by the good surviva-

bility of this virus. In neutral buffered liquids such as cell culture medium BHV1 survives with little or no loss in titre for 15-30 days at 4°C, and with only a small drop in titre in 5-6 days at room temperature (Griffin and others, 1958; Hahnefeld and others, 1963; Drew and others, 1987). Delays in transport of samples to a laboratory are not therefore a barrier to attempted virus isolation. Virus can even be frozen and thawed up to five times with impunity (Stevens and Groman, 1963; Drew and others, 1987).

Appropriate samples are a key to successful diagnosis (Nettleton, 1986) and the material used for sampling must be carefully monitored. Calcium alginate wool swabs are favoured for bacteriological specimens, but are virucidal to BHV1 (Doherty and others, 1967; Hanson and Schipper, 1976).

Terpstra (1979) showed that direct immunofluorescence could be used to detect BHV1 antigen in cellular material in smears of nasal mucus, or in cryostat sections of respiratory epithelial tissues, the tonsil being the tissue of choice. The selection of appropriate cases from an outbreak was critically important: they should be early febrile cases with a serous nasal discharge but before the development of a mucopurulent discharge. In experimentally infected cattle immunofluorescence on nasal smears gave a positive diagnosis from day 2 to day 7 after inoculation, compared with days 1 to 11 by virus isolation in cell culture. Subsequent reports (Silim and Elazhary, 1983; Nettleton and others, 1983) confirmed the value of immunofluorescence for BHV1 antigen detection as a diagnostic test on field material.

Antigen detection by enzyme-linked immunosorbent assay (ELISA) has also been used as a diagnostic test for IBR, with either polyclonal

sera (Nettleton and others, 1982) or monoclonal antibodies (Collins and others, 1985a). In the latter case the sensitivity of the ELISA was equivalent to $10^{3.5}$ $TCID_{50}$ per sample well, or 1.5 ng purified BHV1. Nevertheless it could still detect infection only up to day 5 or 6 after experimental infection, compared with up to day 11 for virus isolation in cell culture. None of the available antigen detection techniques is sufficiently sensitive for diagnostic application during the second week after infection, even though this is the period when many cases are most apparent clinically (Edwards and Gitao, 1987).

A new approach to virus diagnosis is provided by recent developments in DNA technology. The hybridization of labelled DNA probes with viral nucleic acids in samples, either *in situ* in cell or tissue preparations or by blotting on nitrocellulose filters, provides a virus detection technique of potentially high specificity and sensitivity although not, as yet, rivalling the rapidity of antigen detection methods. Because hybridization requires neither replication of the virus nor expression of antigens, it can also detect latent viruses as shown with BHV1 by Lawrence (1983). The general application of hybridization to virus diagnosis was reviewed by Pagano (1982) and Petterson and Hyypia (1985). When applied to herpesvirus diagnosis, 5pg viral DNA could be detected in clinical specimens (Schuster and others, 1986). Dorman and others (1985) compared a variety of biotinylated cloned BHV1 DNA fragments as diagnostic probes for sample DNA extracted and blotted onto nitrocellulose. In samples from naturally infected cattle, the sensitivity compared with virus isolation was 68% and the specificity 95%. Dunn and others (1986) extended this work to *in situ* hybridiza-

tion to cell cultures, and nasal epithelial and buffy coat cells from BHV1 infected cattle, immobilized on glass slides. Pacciarini and others (1986) used radiolabelled probes to detect BHV1 DNA in non-extended semen samples with a sensitivity of 2.5×10^5 virus particles, or 40pg viral DNA.

Detection of host response

With the limited exception of intradermal hypersensitivity tests (Aguilar-Setién and others, 1983) CMI assays have not found applications in diagnosis. In contrast, a wide variety of serological assays for BHV1-specific antibody has been used diagnostically including serum-virus neutralization, passive haemagglutination, gel immunodiffusion, complement fixation, and indirect immunofluorescence (reviewed by Gibbs and Rweyemamu, 1977). More recently, ELISA has been increasingly exploited as a rapid, sensitive and reliable technique (Payment and others, 1979; Herring and others, 1980; Solsona and others 1980; Bolton and others, 1981).

The virus neutralization (VN) test has been very widely used and is the internationally recognized standard procedure (Carbrey and others, 1971; Office International des Epizooties, 1986). Nevertheless a number of workers have commented on the limited sensitivity of the method in comparison with other assays such as passive haemagglutination (Vengris and Maré, 1971; Kirby and others, 1974) or ELISA (Payment and others, 1979; Bolton and others, 1981; Cho and Bohac, 1985; Collins and others, 1985c). Others have modified the conditions of the VN assay to increase its sensitivity: for example, by addition of complement (Rossi and Kiesel, 1974; Potgieter, 1975), reducing challenge virus dose (Bitsch, 1970; Huck and Woods, 1972;

Darcel, 1975) addition of a second dose of challenge virus (Lüpcke and Göbel, 1979), use of constant serum-varying virus (House and Baker, 1971), and prolongation of the neutralization reaction (Bitsch, 1978). The last technique, using a serum-virus reaction time of 24 hours at 37°C was shown by Edwards and others (1986b) to be specific and sensitive and was recommended as a standard procedure, although the ELISA had a similar sensitivity and was recommended for routine diagnostic application, in view of its speed and simplicity.

The technique of ELISA was first described by Engvall and Perlman (1971), and applied to viral serology by Voller and Bidwell (1975). Since then it has found widespread application in diagnostic virology. There have been numerous reports of its use for the detection of BHV1-specific IgG. Although, in the developmental stages of the test, purified viral antigens were generally used to coat microtitre plates (Payment and others, 1979; Solsona and others, 1980; Bolton and others, 1981) others have found that semi-purified antigens (Herring and others, 1980; Cho and Bohac, 1985; Durham and Sillars, 1986) or even crude cell culture lysates (Bommeli and others, 1980) were adequate for diagnostic work. Collins and others (1985c) and Liauw and Eugster (1986) found that, although purified antigens gave a slightly more sensitive test, the extra expense and effort involved could not be justified for routine diagnostic applications in view of the observation that crude, simply prepared antigens gave a test of adequate sensitivity and specificity which correlated well with the VN test. Riegel and others (1987) achieved high sensitivity and specificity in ELISA by competing test sera with a neutralizing monoclonal antibody for binding to BHV1 antigen. The ELISA method has also been adapted to the detection of BHV1 antibodies in milk (Stuker and

others, 1980; Perrin and others, 1984) with the implied possibility of testing bulk milk samples as a screening test for infected herds. The best sensitivity for bulk milk tests reported so far was the detection of one seropositive cow among 50 seronegatives (Forschner and Bünger, 1986; Forschner and others, 1986; Wizigmann, 1987).

Chapter 2

MATERIALS AND METHODS

INTRODUCTION

Many of the specific materials and methods used are described in the following chapters alongside the experiments to which they relate. Those of more general application are presented here.

BUFFERS AND MEDIA

0.01M phosphate buffered physiological saline (PBS)

	pH7.2	pH7.6
Sodium dihydrogen phosphate ($NaH_2PO_42H_2O$)	0.36g	0.20g
Disodium hydrogen phosphate ($Na_2HPO_42H_2O$)	1.37g	1.55g
Sodium chloride (NaCl)	8.5g	8.5g
Distilled water	to 1L	to 1L

For immunofluorescence work, pH7.6 was used. For enzyme immunoassays and general applications pH7.2 was used. For some of the ELISA work equivalent molar concentrations of potassium phosphates were used in the interests of standardization with other workers in the laboratory.

Antibiotic Supplement

100 IU penicillin, 100µg streptomycin, 20 IU mycostatin per ml.

Swab elution medium (PBSL)

PBS pH7.2	85ml
Lactalbumen hydrolysate 5%	10ml
Antibiotic supplement	5ml

Earle's balanced salt solution (10x concentrate)

Sodium chloride	680g
Potassium chloride	40g
Magnesium sulphate ($Mg\ SO_4.7H_2O$)	20g
Sodium dihydrogen phosphate ($NaH_2PO_42H_2O$)	15.6g
Calcium chloride ($CaCl_22H_2O$)	23.4g
Glucose	100g
Yeast extract	10g
Phenol Red 0.2%	500ml

Cell culture medium 1 (EYL)

Earle's balanced salt solution (10x concentrate) (with yeast extract)	100ml
Lactalbumen hydrolysate 5%	100ml
Sodium bicarbonate 7.5%	25ml
Antibiotic supplement	5ml
Sterile deionised water	to 1L

Cell culture medium 2 (MEM)

Eagle's minimal essential medium (10X concentrate, Flow laboratories)	100ml
Glutamine 0.2M	10ml
Sodium bicarbonate 7.5%	20ml
Antibiotic supplement	5ml
Sterile deionised water	to 1L

For cell growth, fetal calf serum was added to 10%, and for cell maintenance to 2% v/v.

0.05M carbonate coating buffer

Sodium carbonate 0.2M	79ml
Sodium bicarbonate 0.2M	171ml
Distilled water	to 1L

pH adjusted to 9.6

Buffered saline with 0.05% Tween (PBST)

PBS pH7.2	1L
Polyoxyethylene glycol (20) sorbitan monolaurate (Tween 20)	0.5ml

ELISA diluent for sera and conjugates

$NaH_2PO_4.2H_2O$	0.36g
$Na_2HPO_4.2H_2O$	1.37g
NaCl	28.95g
Tween 20	0.5ml
Ethylene diamine tetracetic acid (EDTA) 0.1M	10ml
Egg albumen (Sigma)	5g
Distilled water	to 1L

pH adjusted to 7.2

Phosphate-citrate buffer for ELISA substrate

Citric acid 0.1M	243ml
Na_2HPO_4 0.2M	257ml
Distilled water	500ml

pH adjusted to 5.0

ELISA substrate (OPD)

o-phenylene diamine	0.2g
Phosphate-citrate buffer pH5	500ml

The solution was stored frozen at -20°C in the dark in 25ml aliquots. Immediately before use these were thawed to room temperature and 10µl hydrogen peroxide 30% was added per 25ml.

Immunoperoxidase substrate (DAB)

Di-amino benzidine tetrahydrochloride	5mg
PBS pH7.2	10ml
Hydrogen peroxide 30%	5µl

The solution was prepared freshly immediately before use.

VIRUS CULTURE AND TITRATION

Stocks of BHV1 strains were prepared in primary calf kidney cell monolayer cultures. These are prepared on a weekly basis at the Central Veterinary Laboratory (CVL) and checked retrospectively (after passage) for the absence of contaminating BVDV. Known BHV1 positive material was inoculated onto a confluent monolayer at the rate of 1-2ml per 180sq.cm. culture flask. After incubating at 37°C for 1 hour the culture was washed with fresh medium then overlaid with EYL or MEM with 2% fetal calf serum, and incubated at 37°C. When the cytopathic effect was advanced to the point of detachment of most or all of the cells (usually in 2-3 days), the cells were lysed by a freeze-thaw cycle at -70°C and the harvest clarified by centrifugation at 1000g for 15 minutes. The harvest was stored in small aliquots at -70°C.

The identity of isolates and virus stocks was confirmed by immunofluorescence using BHV1-specific conjugate on cultures grown on flying coverslips in Leighton tubes and fixed in acetone at the stage of early cytopathic effect.

Stocks of BHV1 isolates were titrated by preparing a 10-fold dilution series in MEM with 10% fetal calf serum, and transferring each dilution into 4 replicate wells in a flat bottomed tissue culture grade 96 well microtitre plate, 50µl per well. A suspension of Madin-Darby bovine kidney (MDBK) cells was prepared by trypsinization of a confluent monolayer, resuspending the cells to a concentration of 3 x 10^5 per ml in MEM with 10% fetal calf serum. 50µl per well of cell suspension was added to the microtitre plate which was sealed and incubated at 37°C for 5 days. The cells formed a monolayer on the bottom of the wells, and growth of the virus could be assessed by microscopic observation of the cytopathic effect. The titres were calculated as 50% infectious doses ($TCID_{50}$) by the Spearman-Kärber method.

For titrations where large numbers of samples were to be processed, as with the swabs from the experimental calves, 10-fold dilution series from the swab eluting medium were made directly in the microtitre plates using a transfer volume of 10µl into a volume of 90µl MEM per well. For this purpose, 12-channel micropipettes (Flow Laboratories) were used, with a change to fresh sterile pipette tips for each dilution step.

SERUM-VIRUS NEUTRALIZATION TESTS

A constant virus varying serum method was used as described by Frerichs and others (1982). Doubling dilutions of sera were made, in quadruplicate 25µl aliquots, in 96 well flat bottomed microtitre plates using MEM with 10% fetal calf serum as the diluent. BHV1 (Oxford strain) was added at the rate of 100 $TCID_{50}$ in 25µl per well.

The mixture was incubated for 1 hour at 37°C. MDBK cell suspension

was then added as described under virus titration. After 5 days incubation the growth of virus was assessed by microscopic observation of the cytopathic effect. The 50% neutralizing end point titres were calculated by the Spearman-Kärber method, and expressed as the serum dilution before addition of the virus.

ELISA FOR SEROLOGY

For the preparation of antigen, BHV1 (Oxford strain) was grown in cell cultures of primary calf kidney or of MDBK cell line to the point of advanced cytopathic effect. Remaining adherent cells were scraped off then the cells pelleted from the culture medium by centrifugation at 1000g for 10 minutes. The supernatant was discarded and the pellet resuspended in a small volume (1ml per 180 sq. cm. flask or 2ml per roller bottle) of 0.5% polyoxyethyleneglycol (9) p-t-ocytl phenol (Nonidet P40) in PBS. After 1 hour at 4°C the suspension was clarified by centrifugation and the supernatant reserved in small aliquots at -70°C as the antigen. Control antigen was prepared in parallel from non-infected cultures. The antigens were titrated in the ELISA system to determine the optimum coating dilution, which was approximately at the upper shoulder of the sigmoid dose-response curve obtained at a fixed level of positive test serum. This dilution was usually in the range 1/500 to 1/4000 depending on the batch of antigen.

The antigens were diluted in carbonate buffer for addition to 96 well, polyvinyl microtitre plates (Falcon 3912, Microtest III, Becton Dickinson & Co.) at 100µl per well with alternate rows of virus and control antigen. After overnight adsorption at 4°C the plates were washed three times in PBST, then stored at -20°C.

Test sera were diluted 1/100 in ELISA diluent then 50µl was added to

two viruses and two control antigen coated wells. The plates were incubated for 2 hours at room temperature in a humid chamber, then washed five times in PBST. For IgG detection, 50µl per well of rabbit anti-bovine IgG peroxidase conjugate was added at optimal dilution (1/500) in ELISA diluent and the plates incubated for 1 hour at room temperature. They were washed a further five times in PBST before the addition of 100µl per well OPD substrate. The reaction was stopped after 15 minutes by the addition of 25µl per well 2.5M sulphuric acid. The optical densities (OD) were measured on a Multiskan photometer (Flow Laboratories) at 492nm wavelength. The calculations were initially done by hand and latterly by on-line data capture to an Apple IIe microcomputer, for which an appropriate program was written in Applesoft Basic. For each sample, the mean OD of the two replicate wells with control antigen was subtracted from the mean of the two with viral antigen to give the test result. Where the replicates for either virus or control antigens differed by more than 20%, the sample was retested.

For immunoglobulin class or subclass specific tests a similar procedure was followed except that the conjugate stage was replaced with a two stage process involving rabbit anti-bovine heavy chain specific (IgM, IgG1, IgG2 or IgA) antisera (Miles Scientific) used at dilutions in ELISA diluent of 1/100, 1/400, 1/200 and 1/100 respectively for 1 hour. After five washes in PBST, protein A - peroxidase conjugate (Sigma) was added to the wells at 125 pg/ml for 30 minutes, washed five times in PBST then substrate added as above.

Standard sera for use in the assays were prepared by the inoculation of calves with BHV1 intranasally and bleeding at 10 days (IgM control) and 6 weeks post inoculation (IgG control).

PREPARATION OF HYPERIMMUNE SERA

Gnotobiotic antiserum to BHV1 was provided by Miss M Lucas. It had been prepared in a hysterotomy-derived calf maintained in a sterile environment under positive pressure ventilation. The calf was inoculated with semipurified (pelleted) BHV1 Oxford strain, initially intranasally, two weeks later intranasally and intramuscularly and, at 4 and 8 weeks after the initial dose, intramuscularly and intraperitoneally. It was killed and bled out one week after the final boost, when it had a VN titre of 1/256.

For general diagnostic work, including the preparation of bulk stocks of virus-specific fluorescent antibody conjugates, a colostrum-deprived calf was inoculated intranasally with BHV1, maintained in isolation for two months then boosted with virus by the intravenous route. Serum was collected 11 days later and had a VN titre of 1/256.

For the preparation of rabbit antisera to bovine immunoglobulin, IgG was purified from bovine serum by the batch method using DEAE-cellulose (DE52, Whatman) (Reif, 1969). 200µg IgG was dissolved in 1 ml PBS and emulsified with 1 ml complete Freund's adjuvant (Difco). Rabbits were inoculated with 0.25 ml emulsion at each of four intramuscular sites. The inoculation was repeated three weeks later and serum collected one to two weeks after that.

PREPARATION OF FLUORESCENT ANTIBODY CONJUGATES

The method was based on that of Nairn (1976). Immunoglobulins were precipitated from hyperimmune antisera of the desired specificity by the addition of crystals of sodium sulphate (1.4g per 10ml serum), stirring for 2 hours, then centrifugation at 1000g for 20 minutes, all at room temperature. The precipitate was dissolved in PBS pH7.2 then

reprecipitated and dissolved two more times. The final solution was dialyzed against PBS overnight at 4°C. The protein concentration was estimated from the absorbance at 280nm wavelength using the formula:

$$\text{Concentration (mg/ml)} = \frac{\text{Absorbance} \times 10 \times \text{dilution factor}}{E}$$

where the extinction coefficient (E) was 13.7 for bovine IgG and 15.0 for rabbit. The concentration was adjusted to 10mg/ml. Carbonate buffer 0.5M pH9.0 (3.7g $NaHCO_3$, 0.6g anhydrous Na_2CO_3, 100ml distilled water) was added in the proportion 1 volume to 2 volumes protein solution. Fluorescein isothiocyanate (FITC, isomer 1, Becton Dickinson) was added, with stirring, at the rate of 1mg per 60mg protein. The mixture was stirred gently overnight then extensively dialyzed against physiological saline (0.14M) buffered to pH8.7 with 0.01M Tris-hydrochloric acid. The conjugate was separated from unconjugated material, initially by gel filtration on a column of Sephadex G25 (Whatman) using the same Tris buffered 0.14M saline, then by ion exchange chromatography on a DEAE-cellulose column (DE-23, Whatman) eluting fractions with 0.14M and 0.28M Tris buffered saline. Conjugates were stored in small aliquots at -20°C. The working dilution (in the range 1/4 to 1/16) was determined by titration on known positive and negative materials.

PREPARATION OF PEROXIDASE CONJUGATES

IgG was purified from hyperimmune antisera of the desired specificity by the batch method (Reif, 1969). For each 1ml of serum 6g of DE52 was equilibrated in 0.01M phosphate buffer pH7.4, washed and dried under suction in a Buchner funnel. The serum was dialyzed against the same buffer, diluted in it 1/3 and mixed with the equilibrated DE52. The mixture was kept on ice for one hour with intermittent stirring. The liquid phase was extracted by suction and washed out

with more of the buffer. The IgG in the eluate was concentrated by precipitation with sodium sulphate (see above), redissolution and dialysis against 0.01M sodium carbonate buffer pH9.5. The protein concentration was estimated from the absorbance at 280nm (see above) and adjusted to 8mg/ml.

The conjugation method was based on that of Wilson and Nakane (1978). 4mg horseradish peroxidase (Sigma type VI) was dissolved in 1ml distilled water, then 0.2ml freshly prepared 0.1M sodium meta-periodate was added and stirred for 20 minutes at room temperature. The solution was dialysed overnight against 1mM sodium acetate buffer pH4.4 at 4°C. The next day the pH was raised by the addition of 20µl 0.2M sodium carbonate buffer pH9.5, and 1ml of the protein solution was added. The mixture was stirred for 2 hours at room temperature then 0.1ml freshly prepared sodium borohydride 4mg/ml was added and left for 2 hours at 4°C. This conjugate was used without further purification and was stored in 50% glycerol solution at -20°C.

Chapter 3

TRENDS IN NATURALLY OCCURRING DISEASE ASSOCIATED WITH BOVID HERPESVIRUS 1 IN GREAT BRITAIN.

INTRODUCTION

Epidemiological data on BHV1 infection and its associated diseases in Great Britain are incomplete. Early papers were presented as case reports of IBR (Dawson and others, 1962; Darbyshire and Shanks, 1963) and IPB/IPV (Huck and others, 1971; Collings and others, 1972). Serological surveys gave estimated seroprevalence for the virus as 2.1% (Dawson and Darbyshire, 1964), 6.8% (sampled in 1974) (Kirby and others, 1978) and 12% (sampled in 1977) (D H Roberts, personal communication, summarized by Virology Dept., 1978). The three values should not be compared quantitatively as different methods of measuring the antibody were used (VN in tubes, indirect haemagglutination, and VN in microplates respectively).

Beginning in the winter of 1977-78, widespread reports (summarized by Epidemiology Unit, 1979) indicated a sudden rise in the incidence and clinical severity of IBR, typically associated with high herd morbidity and variable but significant mortality. This was supported by clinical and pathological studies from the University of Glasgow (Wiseman and others, 1978; Wiseman and others, 1980; Allan and others, 1980). Subsequently, Msolla and others (1981) suggested that the BHV1 seroprevalence in Scotland had increased (to 12%) over earlier reports; notably in herds which had introduced Holstein cattle. They did not state the year in which the samples were collected. A similar prevalence (11.4%) was reported by Peters and Perry (1983) for bulls

at performance testing stations in the period 1979-1981. Further developments, likely to influence the disease epidemiology, occurred in 1979 and 1981 with the announcement of product licences and commercial availability of live attenuated intranasal BHV1 vaccines (Tracherine, Smith Kline Animal Health [Veterinary Record 105 : 340] and Nasalgen, Wellcome [Veterinary Record 109 : 170]).

In an attempt to gain an overall view, particularly for the period 1970-1986, data from the records of MAFF veterinary laboratories and Scottish Agricultural Colleges have been further analysed and are presented in this chapter.

MATERIALS AND METHODS

Three sources of data were used. None represents an unbiased sample of the cattle population and two were derived from the same source material - namely clinical incidents presented to the Veterinary Investigation (VI) Service for disease investigation and diagnostic laboratory testing. The material had been selected initially by the farmer, in choosing to consult his veterinary surgeon, and subsequently by the veterinary surgeon in choosing to avail himself of the Veterinary Investigation services.

1. VIDA II

The Veterinary Investigation Diagnosis Analysis - Mark II (VIDA II) (Hall and others, 1980) commenced in 1975 and comprised over 2.2 million computerised records by the end of 1986. The data were supplied by Veterinary Investigation Centres in England, Scotland and Wales. The VIDA II annual report (MAFF and others, 1987) cautions about the inherent bias in the data and the need to relate trends for

individual diseases to the figures for total incidents recorded. BHV1-associated disease was not recorded as a specific diagnosis on VIDA II until 1980, when it appeared under "Group 1: Systemic diseases of cattle and those not readily classified organically" as "IBR/IPV". Additional entries were made in "Group 9: Diseases of the reproductive and mammary systems of cattle" as "Foetopathy *(sic)* due to IBR/IPV" The data were analysed temporally (by month and year) and geographically (by county or region).

2. CVL, Virology Department diagnostic testing

Materials for diagnostic testing were received at the CVL only via the Veterinary Investigation Service. All incidents investigated at the CVL were therefore also recorded on VIDA. Nevertheless, more detailed records were available at the CVL than were entered on VIDA and a separate analysis was carried out. Conversely, not all VIDA incidents were investigated at the CVL - during the study period, an increasing number of diagnostic tests for BHV1 were carried out in Veterinary Investigation Centres without further referral, whilst in Scotland the majority of tests were carried out at the Moredun Institute in Edinburgh. Nevertheless, sufficient testing was done at the CVL to warrant analysis of the data.

Information on the number of tests done and number of positive diagnoses was available, through the Virology departmental annual reports, from 1969. In addition, from 1977 onwards detailed case records were kept of all incidents in which a diagnosis of active BHV1 infection was considered to be established, either by virus isolation/antigen detection or by seroconversion. These records included laboratory reference numbers, date, VI centre of origin,

basis of the laboratory diagnosis, and as much of the clinical and pathological history as was supplied on the sample submission document. The history varied from nothing at all to a fully detailed account of the incident. The data in this case records file was subsequently reduced to a codified form and entered into a database using Prime Information software on the CVL Prime minicomputer. The data format is shown in Table 3.1. Analysis was carried out on-line using the Inform query language, which forms part of the software system.

3. CVL, Virology Department statutory and export testing

Serum samples are submitted directly to the CVL by veterinary practitioners and by artificial insemination (AI) organisations for screening for BHV1 antibodies, the result being purely qualitative, ie positive or negative. The samples are from healthy animals, either prior to export or prior to entry to AI studs. The data are not recorded on VIDA and represent a section of the cattle population different from the previous analyses, ie predominantly pedigree breeding herds and, in the case of the AI samples, males only.

Diagnostic test methods are continuously subject to review and modification, which may lead to changes in apparent prevalence and incidence rate over a period of years, with the inherent risk of false assumptions about the disease epidemiology. In particular, two major changes in serological procedures for BHV1 diagnosis were made at the CVL during the period under review, in each case involving an increase in test sensitivity and therefore the possibility that more seropositive animals were detected. Until April 1976 the virus neutralization test was carried out in test tube cell cultures using 0.1 ml per tube of serial serum dilutions mixed with an equal volume

of medium containing 100 $TCID_{50}$ BHV1 as described by Dawson and others (1962). From April 1976 until August 1985 the VN test was done in the microtitre plate system using 0.025 ml of serum dilution and 100 $TCID_{50}$ virus per test well following the procedure described by Frerichs and others (1982). The method is still in use for most export tests. Some export tests and all AI and routine diagnostic tests have been done by ELISA since August 1985, using the method described in Chapter 2.

4. Statistical analysis

Frequency distributions of the incidence and prevalence of BHV1 infections were analysed by the chi-squared technique for one or two dimensional tables as appropriate. Secular trends were expressed graphically by the use of rolling averages (Thrusfield, 1986b).

RESULTS

1. General

4724 incidents of IBR/IPV were recorded on VIDA II for the period 1980-86, the majority (4372) under the heading of systemic diseases and the remainder (352) as fetopathies. At the CVL, serological investigations for BHV1 were carried out on paired sera from 2656 incidents of respiratory disease in the period 1971-1986. Rising antibody titres to BHV1 were shown in 426 (16%) of the incidents. Similar figures for reproductive disease for 1970-1986 were 2086 incidents investigated, of which 158 (7.6%) had rising titres to BHV1. Submissions of tissues and swabs for virus isolation could not be separated by disease category but BHV1 was isolated from 513 of the total 19,189 disease incidents investigated in the period 1969-1986. An unquantifiable proportion of these incidents was also investigated

by serology and therefore included in both totals. Separate successive submissions of samples to the CVL from the same disease incident in any one herd could not be identified from the available data. Such submissions were therefore recorded as separate incidents for the CVL analysis although recorded only once in the VIDA II analysis. Evidence from the CVL case records analysis, for which this information was available, suggested that this accounted for only a small proportion of over-reporting. The case record analysis included 888 BHV1associated clinical incidents, in the decade 1977-86.

Testing of bulls for BHV1 antibody before entry to AI centres commenced in 1970. 26,585 animals were tested up to the end of 1986, of which 2716 (10.2%) were positive. For the period 1969-1986, 61,257 cattle were tested for BHV1 antibody for export certification purposes; of these 5447 (8.9%) were positive.

2. Temporal analysis

The distribution of IBR/IPV incidents recorded on VIDA II is shown in table 3.2. There was a highly significant variation ($P<0.001$) between monthly totals and between yearly totals for both systemic disease and fetopathy. The overall distribution for systemic disease also showed a significant variation ($P<0.001$) when analysed as a 12 x 6 contingency table. The chi-squared test was not valid for the latter analysis on fetopathy incidents due to the low frequencies in many cells. The seasonal and annual trends are shown graphically in fig 3.1, which also shows that a similar seasonal trend was observed in the CVL case records analysis.

A longer annual analysis, embracing the important period in the 1970s when the IBR disease pattern was changing, was possible from the CVL,

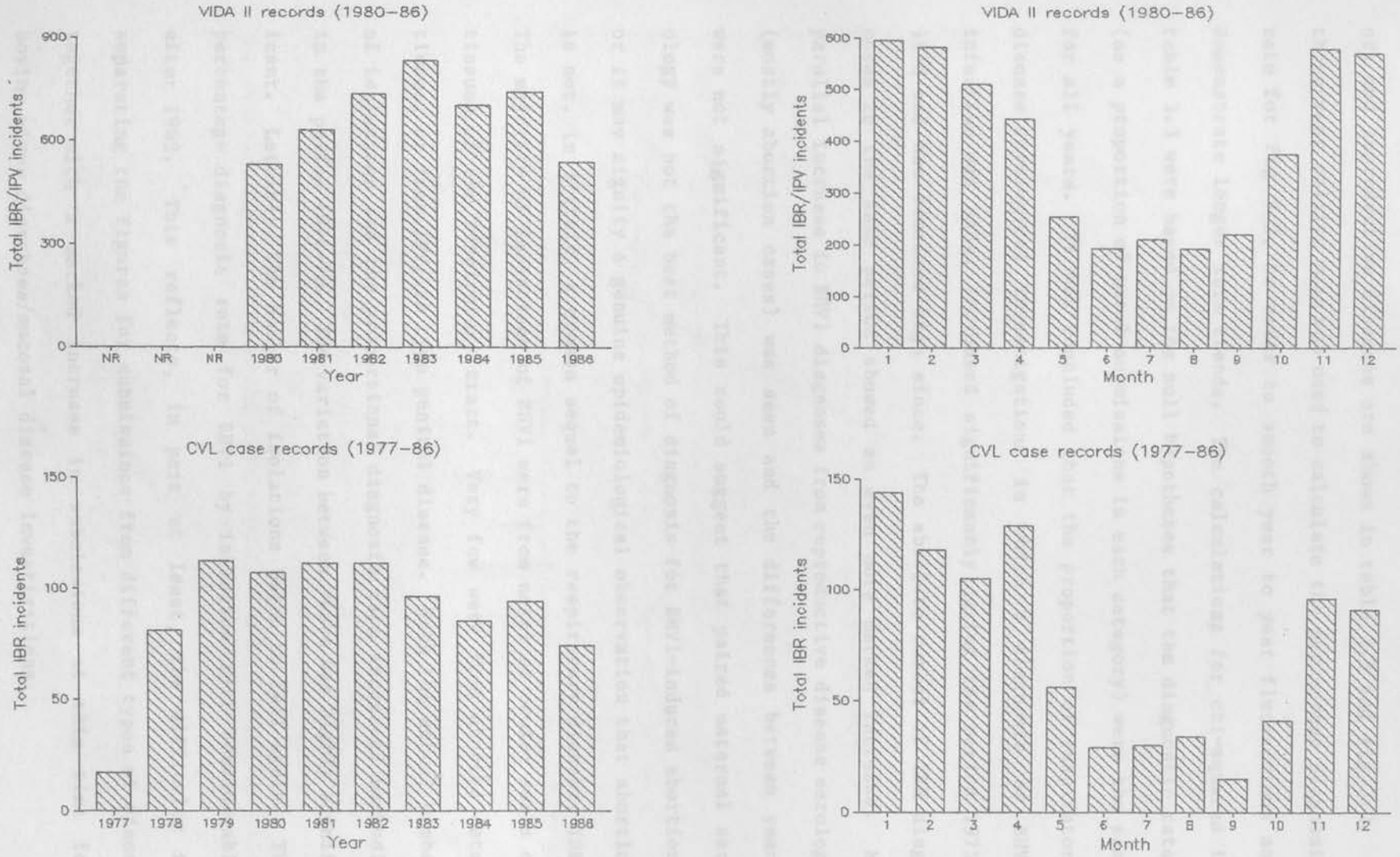
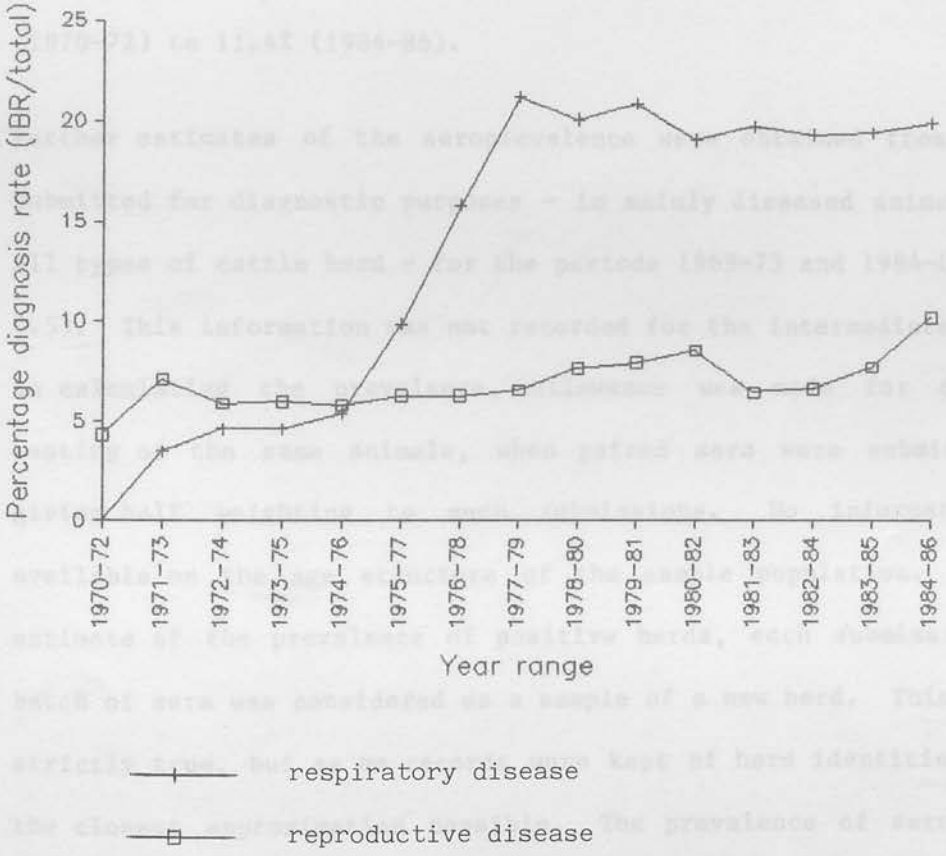


Fig. 3.1 Yearly and seasonal trends in IBR incidence

Virology Dept archives. The results of diagnostic tests for respiratory and reproductive diseases are shown in table 3.3 and fig 3.2. A three-year rolling mean was used to calculate the percentage diagnosis rate for fig 3.2, in order to smooth year to year fluctuations and demonstrate longer term trends. The calculations for chi-squared in table 3.3 were based on the null hypotheses that the diagnostic rates (as a proportion of total submissions in each category) were the same for all years. It was concluded that the proportion of respiratory disease serological investigations in which a diagnosis of BHV1 infection was made, increased significantly during the period 1975-1978 and has remained high since. The absolute number of IBR diagnoses in the same period showed an even more marked increase. No parallel increase in BHV1 diagnoses from reproductive disease serology (mostly abortion cases) was seen and the differences between years were not significant. This could suggest that paired maternal serology was not the best method of diagnosis for BHV1-induced abortion, or it may signify a genuine epidemiological observation that abortion is not, in Britain, a common sequel to the respiratory disease, IBR. The majority of isolations of BHV1 were from nasal or ocular swabs or tissues of the respiratory tract. Very few were from aborted fetal tissue, or from IPV/IPB type genital disease. Both the actual number of isolations and the proportional diagnosis rate increased markedly in the period 1976-79. The variation between years was highly significant. Latterly, the number of isolations began to fall again. The percentage diagnosis rate for BHV1 by isolation fell considerably after 1982. This reflects, in part at least, the difficulty in separating the figures for submissions from different types of disease, together with a marked increase in submissions at this time for bovine virus diarrhoea/mucosal disease investigations.



The data are the percentage of submissions of paired sera in which rising titres to BHV1 were found, expressed as 3-year rolling means.

Fig. 3.2 Proportional BHV1 diagnosis rate for bovine respiratory and reproductive diseases tested by paired serology at the CVL

Serum samples tested at the CVL are not randomly selected nor are they representative of any particular segment of the cattle population. Nevertheless they were used to give some idea of the prevalence of BHV1 seropositive animals. The most complete set of data is for healthy pedigree animals tested for AI or export purposes (Table 3.4). It was considered that the two groups were sufficiently similar in origin to produce combined values for the prevalence. The chi-squared test for the null hypothesis that the prevalence had not changed was highly significant. The long term trends indicated by the 3 year rolling mean (Fig. 3.3) showed an increase from 4.7% (1970-72) to 11.4% (1984-86).

Further estimates of the seroprevalence were obtained from samples submitted for diagnostic purposes - ie mainly diseased animals, from all types of cattle herd - for the periods 1969-73 and 1984-86 (Table 3.5). This information was not recorded for the intermediate period. In calculating the prevalence, allowance was made for duplicate testing of the same animals, when paired sera were submitted, by giving half weighting to such submissions. No information was available on the age structure of the sample population. For the estimate of the prevalence of positive herds, each submission of a batch of sera was considered as a sample of a new herd. This was not strictly true, but as no records were kept of herd identities it was the closest approximation possible. The prevalence of seropositive animals and of herds containing seropositive animals showed significant variation between years ($P<0.001$). The most marked feature was a rise in mean animal prevalence from 9.1% in the period 1969-73 to 34.7% for 1984-86; and a corresponding increase in prevalence of seropositive herds from 17.6% (1970-73) to 48.0% (1984-86).

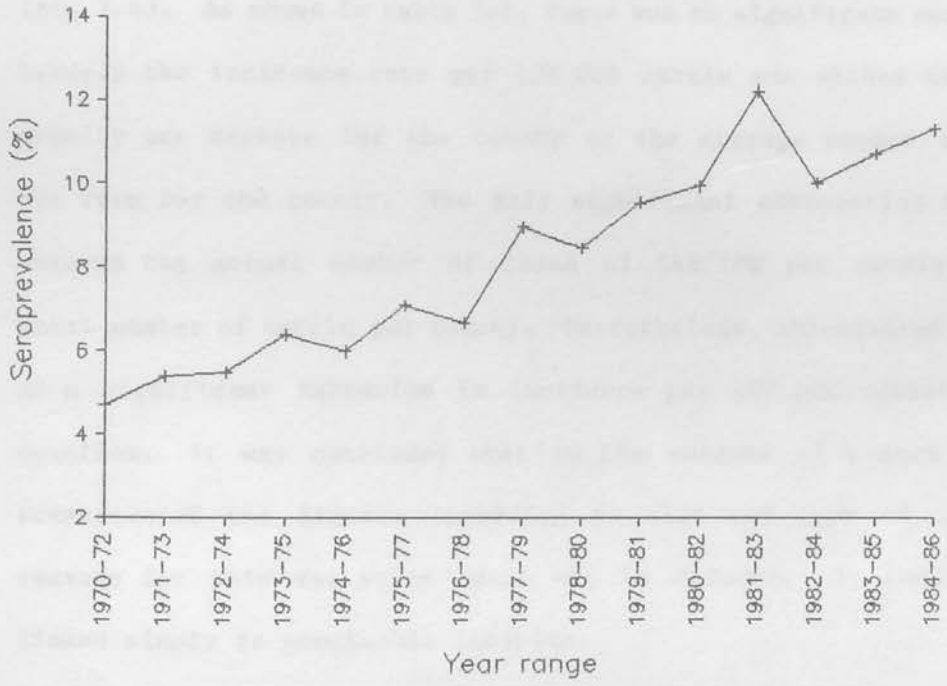


Fig. 3.3 Percentage seroprevalence of BHV1 antibodies in healthy pedigree cattle tested at the CVL (3-year rolling means)

3. Geographical analysis

The frequency distribution of IBR/IPV incidents by county, from the VIDA II records, is shown in appendix 3 (i) and (ii) together with census statistics for England (MAFF, 1983), Wales (Welsh Office, 1984) and Scotland (DAFS, 1985).

There may be some discrepancies in the Scottish data as VIDA returns were made for the traditional counties whereas the census statistics were given for the modern regions or for broader geographical zones. There were no consistent geographical patterns for IBR/IPV incidence (Fig 3.4). As shown in table 3.6, there was no significant correlation between the incidence rate per 100,000 cattle and either the cattle density per hectare for the county or the average number of cattle per farm for the county. The only significant correlation found was between the actual number of cases of IBR/IPV per county and the total number of cattle per county. Nevertheless, chi-squared indicated a significant variation in incidence per 100,000 cattle between counties. It was concluded that in the absence of a more detailed breakdown of the figures according to size and type of farm, the reasons for this variation could not be deduced. It could not be linked simply to geographic location.

A second geographical analysis of the VIDA II data, by VI centre, is shown in appendix 3 (iii). Although less informative than the county data, it provides a comparison with the case records file (also summarised in appendix 3 (iii)) for which this was the only geographical information available. The case records data covered mainly England and Wales. The calculation of chi-squared for England and Wales, 1980-86, showed that the distribution of incidents in the

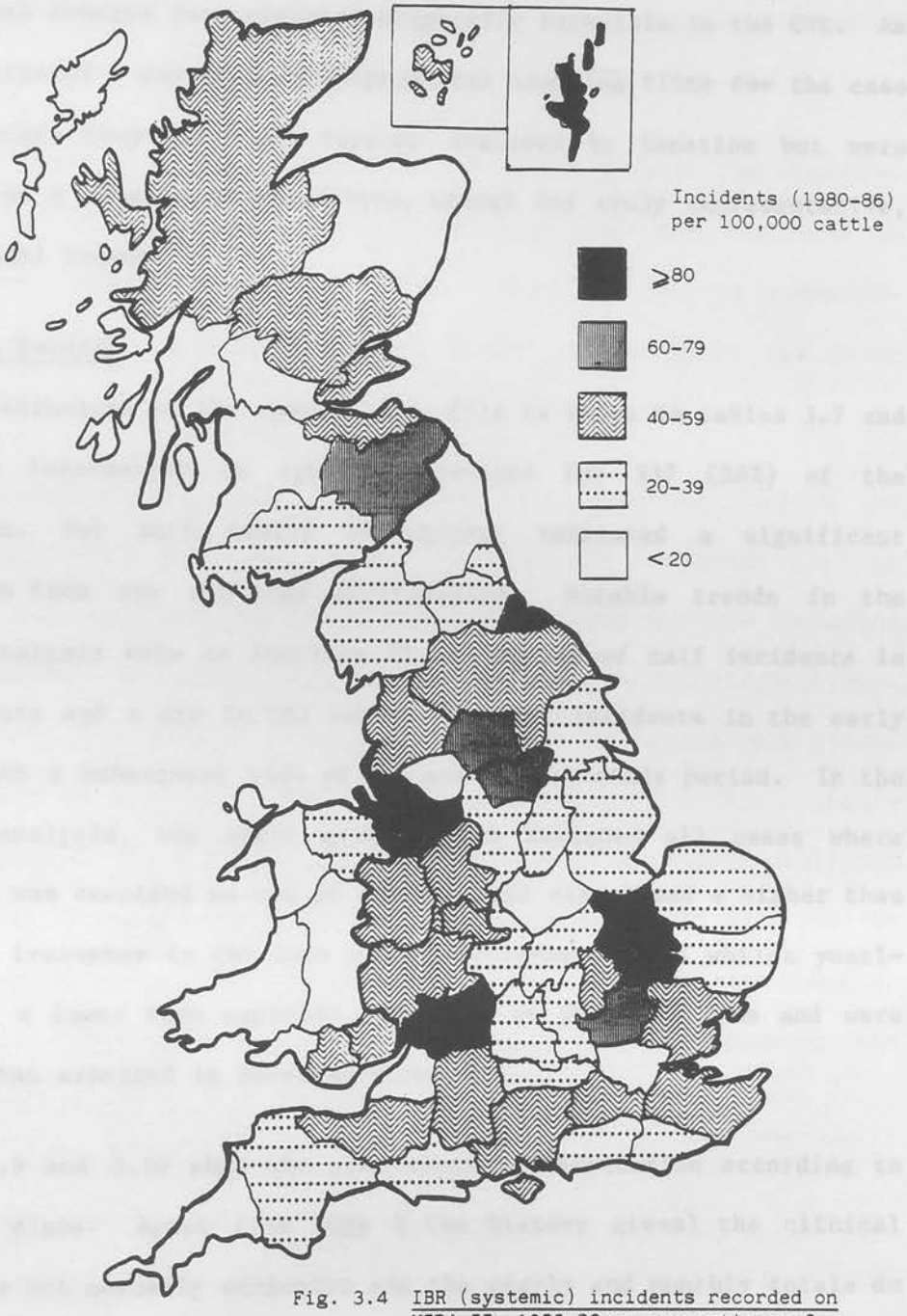


Fig. 3.4 IBR (systemic) incidents recorded on VIDA II, 1980-86 as proportions of county cattle populations

case records file differed significantly from the expected distribution based on the VIDA II returns (χ^2 = 1020.9, df = 24, P<0.001). This was a reflection of the differing policies of individual centres for referring diagnostic materials to the CVL. As this indicated a non-uniform geographical sampling frame for the case records data they were not further analysed by location but were treated as a single set indicative, though not truly representative, of national trends.

4. Case Records

The age structure of the case records file is shown in tables 3.7 and 3.8. No information on age was provided for 337 (38%) of the incidents. For both tables chi-squared indicated a significant variation from the expected distribution. Notable trends in the yearly analysis were an increase in the number of calf incidents in later years and a dip in the number of adult incidents in the early 1980s with a subsequent rise at the end of the study period. In the monthly analysis, the adult group (which included all cases where abortion was recorded as one of the clinical signs) had a higher than expected frequency in the late summer (July-September) whilst yearlings had a lower than expected frequency at the same time and were higher than expected in November-December.

Tables 3.9 and 3.10 show the case records distribution according to clinical signs. Apart from code X (no history given) the clinical codes are not mutually exclusive and the yearly and monthly totals do not represent the sums of each column. Chi-squared was calculated for codes with a total of more than 20 observations. In the yearly analysis (Table 3.9) abortion (code A) ocular disease (E) pneumonia

(L) tracheitis (T) mortality (Z) and no history (X) differed significantly from the expected distribution. The proportion of cases involving abortion rose in later years. The proportion of pneumonia cases was high in the last two years of the study whereas tracheitis and mortality were both relatively more frequent in 1978-79.

Only abortion (code A) differed significantly from the expected seasonal distribution (Table 3.10). It was lower than expected in the winter period (December-April) and higher from July to September. This corresponded with the observed trend for the adult age group (Table 3.8).

Of the 888 cases analysed, laboratory tests used to confirm the diagnosis of BHV1 infection were: serology 460 incidents (417 by serology alone); virus isolation 460 incidents (409 by virus isolation alone); antigen detection 22 incidents (8 by antigen detection alone). Antigen detection by the fluorescent antibody technique was widely used by the VI service to diagnose IBR by testing nasal mucus smears (see Chapter 6) but suitable samples were only occasionally available at the CVL, hence the low figure in this category. The distribution of samples in which BHV1 or its antigen were detected is shown in table 3.11. This indicates the predominance of the respiratory form of BHV1 infection.

SUMMARY AND MAIN CONCLUSIONS

Analysis of BHV1 diagnostic test results from the Virology Dept, CVL (1969-86), case histories from investigations done at the CVL on behalf of the VI service (1977-86), and VI service diagnostic data (VIDA II, 1980-86), using chi-squared to test for significance, have

shown the following trends:

1. BHV1-associated disease had a seasonal incidence. Systemic disease (mainly IBR) predominated from October to April, and fetopathy from July to September.

2. Anecdotal reports of a marked rise in IBR incidence in the late 1970s are supported by the data, which showed the peak incidence in 1983. Fetopathy diagnoses were still rising at the end of the study period.

3. The seroprevalence of BHV1 antibody in cattle also rose, particularly in the period 1977-81, and the number of BHV1 isolates at the CVL increased at the same time. The latest estimates (1986) for seroprevalence were 31% for diseased cattle (sera submitted for diagnosis) and 13% for healthy pedigree cattle (export and AI testing). Estimates from the early 1970s gave values of <10% in both cases.

4. The proportion of herds tested in which one or more BHV1 seropositive animals were found also increased from 16% in 1970 to 44% in 1986.

5. The incidence of BHV1-associated disease per 100,000 cattle varied significantly between counties but could not be related either to the county average cattle density per hectare of agricultural land or to the county average number of cattle per farm. The disease was recorded in all parts of mainland Britain.

6. Case history analysis indicated a changing pattern of BHV1-associated disease in the study period. In the first septennium of the 1980s the incidence in calves (<6 months) and adults (>2 years)

increased, as did the incidence of pneumonic disease and abortion. In contrast, the incidence of the more severe manifestations of upper respiratory disease, exemplified by tracheitis and mortality, decreased during this time.

TABLE 3.1 IBR/IPV case records computer database format

Field name	Data content	Data format
@ID	Case number	7 digits (year + 5 digit reference)
YEAR	Year	2 digits
MONTH	Month when case first reported.	2 digits
VIC	VI Centre submitting samples	2 digit numeric code
DIAG	Laboratory tests used to confirm diagnosis	2 letter code (up to 3 entries per case)
AGE	Age group of animals affected	1 letter code
CLINIC	Coded clinical signs	Up to 8 single letter codes
DNA	Restriction endonuclease type of virus (if known)	1 digit

Diagnosis codes

First letter	Second letter	
S = serology	(R = rising titre	
	(H = high titre (only if considered	
	(diagnostically significant)	
	(M = IgM	
V = virus isolation	(N = nasal swab	E = eye swab
A = antigen detection	(V = vaginal swab	P = preputial swab
	(L = lower respiratory	T = trachea, larynx,
	(tract	pharynx
	(F = fetus or placenta	G = alimentary tract
	(B = cns	H = blood
	(O = others	

Age codes

C = calf (<6 months) Y = "yearling" (6 months-2 years)
A = adult (>2 years) N = not recorded

Clinical codes

A = abortion, B = nervous signs, C = cough, D = diarrhoea/dysentery, E = eye discharge/conjunctivitis, F = fever, G = gastro=intestinal lesions, H = haemorrhagic lesions, I = infertility, L = lung lesions/ clinical pneumonia, M = reduced milk yield N = nasal discharge, rhinitis, and unspecified respiratory signs, P = retained placenta, Q = skin and teat lesions, R = reduced appetite, S = salivation, T = tracheitis, laryngitis, pharyngitis, V = vaginal lesions or discharge, X = no history supplied (but if nasal swab submitted, record as N), Z = deaths.

TABLE 3.2 IBR/IPV incidents recorded on VIDA II

Month	1980	1981	1982	1983	1984	1985	1986	TOTAL
Systemic disease								
Jan	73	74	91	86	85	109	51	569
Feb	74	91	94	74	82	74	55	544
Mar	50	68	71	110	57	68	63	487
Apr	64	52	72	70	61	49	50	418
May	32	33	33	28	30	42	40	238
Jun	29	31	29	23	21	24	14	171
Jul	20	31	18	29	18	26	14	156
Aug	16	24	17	25	21	27	18	148
Sep	17	25	39	32	26	24	19	182
Oct	30	38	54	66	53	70	38	349
Nov	45	62	79	129	102	78	61	556
Dec	67	73	108	108	75	63	60	554
TOTAL	517	602	705	780	631	654	483	4372
Fetopathy								
Jan	0	2	4	1	9	4	6	26
Feb	5	1	8	4	8	4	8	38
Mar	0	2	1	4	5	8	3	23
Apr	2	1	6	4	2	6	3	24
May	0	1	1	2	2	7	3	16
Jun	0	0	0	3	2	7	10	22
Jul	2	2	3	7	12	15	14	55
Aug	1	0	1	4	9	18	12	45
Sep	1	3	1	9	7	7	11	39
Oct	1	0	1	6	8	5	5	26
Nov	1	1	2	2	6	4	6	22
Dec	1	0	2	6	2	1	4	16
TOTAL	14	13	30	52	72	86	85	352
TOTAL CATTLE INCIDENTS ON VIDA II	103098	103305	101962	106638	99377	109728	102249	726357

Contd ...

TABLE 3.2 (Contd)

Statistical analysis

		χ^2	d.f.	P
Systemic disease:	Months (a)	938.22	11	<0.001
	Years (b)	91.59	6	<0.001
	Months x Years	134.64	66	<0.001
Fetopathy:	Months (a)	55.45	11	<0.001
	Years (b)	118.38	6	<0.001
	Months x Years	91.69	66	(<0.05)(c)

Notes (a) Null hypothesis: all months had the same frequency.

(b) Null hypothesis: IBR incidents were a constant proportion of total cattle incidents of VIDA II.

(c) Test unreliable - many cells with low expected frequency.

TABLE 3.3 Summary of IBR/IPV diagnoses at CVL

	Respiratory disease serology			Reproductive disease serology			Virus isolation		
Year	No. incidents investigated[a]	No. incidents positive[b]	Percentage positive	No. incidents investigated	No. incidents postive	Percentage positive	Total bovine submissions	No. incidents where BHV1 isolated	Percentage positive
1964	(310)[c]	(17)							
1969	(310)	(13)		(26)	0	0%	185	3	1.6%
1970	(236)	(4)		23	1	4.3%	147	3	2.0%
1971	155	2	1.3%	15	2	13.3%	122	0	0%
1972	114	5	4.4%	32	0	0%	236	1	0.4%
1973	76	5	6.6%	52	5	9.6%	219	0	0%
1974	72	2	2.8%	51	3	5.9%	348	0	0%
1975	89	4	4.5%	98	4	4.1%	563	0	0%
1976	153	11	7.2%	110	8	7.3%	802	1	0.1%
1977	121	21	17.4%	124	9	7.3%	945	5	0.5%
1978	185	41	22.2%	194	10	5.2%	918	30	3.3%
1979	220	50	22.7%	243	18	7.4%	1053	58	5.5%
1980	175	26	14.9%	151	17	11.3%	1184	73	6.2%
1981	143	37	25.9%	131	7	5.3%	1278	97	7.6%
1982	240	44	18.3%	146	13	8.9%	1685	92	5.5%
1983	305	56	18.4%	278	16	5.8%	2398	55	2.3%
1984	197	45	22.8%	112	7	6.3%	2235	44	2.0%
1985	247	46	18.6%	163	20	12.3%	2703	34	1.3%
1986	164	31	18.9%	163	18	11.0%	2168	17	0.8%
χ^2		86.4			19.7			380.7	
d.f.		15			16			17	
P		<0.001			>0.10			<0.001	

NOTES: (a) For serology, only incidents where paired sera were tested are recorded.
(b) Serology incidents positive: indicates the detection of at least one rising antibody titre to BHV1.
(c) Figures in parenthesis are for numbers of individual animals, where number of incidents was not available.

TABLE 3.4 Summary of BHV1 serological tests on healthy cattle (for AI and export)

Year	Animals tested for AI	No. positive	Animals tested for export	No. positive	Combined prevalence (%)
1969	-	-	766	3	0.4
1970	427	20	503	3	2.5
1971	1091	80	510	16	6.0
1972	1033	66	517	5	4.6
1973	1565	122	862	11	5.5
1974	1379	107	656	19	6.2
1975	1134	108	830	42	7.6
1976	1519	101	2288	91	5.0
1977	1470	156	1472	124	9.5
1978	1822	153	7254	435	6.5
1979	1729	287	12746	1235	10.5
1980	3412	162	8892	787	7.7
1981	1347	223	4754	472	11.4
1982	1435	224	3646	482	13.9
1983	1432	185	1415	142	11.5
1984	3258	226	2886	168	6.4
1985	1629	263	6870	899	13.7
1986	1330	253	4390	513	13.4

χ^2 (17 d.f.) = 1781, P<0.001.

TABLE 3.5 Estimated prevalence of BHV1 seropositive animals and herds, from diagnostic samples submitted to CVL.

	Submissions with paired sera		Submissions with non-paired sera					
Year	Total tests (Tp)	Tests positive (Tpp)	Total tests (Ts)	Tests positive (Tsp)	Estimated seroprevalence (a) (% of animals tested)	Total submissions	Submissions with positive sera	Estimated herd prevalence(b) (% of submissions)
1969	744	65	487	30	7.3	N/A	-	-
1970	679	43	155	17	7.8	134	22	16.4
1971	1748	58	433	20	3.3	251	32	12.7
1972	1286	97	650	89	10.6	122	19	15.6
1973	968	164	331	66	18.2	202	52	25.7
1984	2172	859	2291	991	42.1	941	504	53.6
1985	3073	1120	4578	1429	32.5	1328	649	48.9
1986	1837	687	2388	685	31.1	1431	624	43.6
χ^2		1154		417.5			145.4	
P		<0.001		<0.001			<0.001	

Notes: (a) $\frac{0.5\ (Tpp) + (Tsp)}{0.5\ (Tp) + (Ts)} \times 100$

(b) Not a true herd prevalence, as repeated submissions from the same herds were frequent but unquantified.

TABLE 3.6 Statistical analysis of IBR/IPV incidence for 1980-85 by county (from VIDA II)

SUMMARY OF DATA (No. of incidents)

	Year	1980	1981	1982	1983	1984	1985	1986	TOTAL
IBR/IPV (systemic)	England	409	487	563	617	438	500	354	3368
	Wales	25	26	26	41	50	56	26	250
	Scotland	82	100	108	118	138	97	100	743
IBR/IPV (fetopathy)	England	9	8	19	41	48	71	64	260
	Wales	0	0	1	3	9	5	9	27
	Scotland	5	5	10	9	15	9	15	68

CHI SQUARED TEST ON COUNTY VALUES (six year totals) (from Appendix 3(i) and (ii)):

	Mean No. of incidents	χ^2	d.f.	P
Incidence per 100,000 cattle:				
systemic disease	42.5	1466	65	<0.001
fetopathy	2.7	288	65	<0.001
Incidence per 100,000 hectares:				
systemic disease	32.1	3782	65	<0.001
fetopathy	2.2	507	65	<0.001

CORRELATIONS FOR COUNTY DATA:

	r	n	P
Systemic disease/100,000 cattle vs. cattle/hectare	-0.15	66	>0.05
" " " " vs. cattle/holding	-0.13	58	>0.05
Fetopathy/100,000 cattle vs. cattle/hectare	-0.01	66	>0.05
" " " vs. cattle/holding	-0.02	58	>0.05
Systemic disease incidents vs. No cattle in county	0.73	66	<0.01
Fetopathy incidents vs. No cattle in county	0.65	66	<0.01

TABLE 3.7 Analysis of case records file by age and year

Year	1977	78	79	80	81	82	83	84	85	86	TOTAL
Age group											
Adults	9	31	31	26	22	15	30	25	43	40	272
Yearlings	4	13	21	21	18	21	15	14	13	7	147
Calves	2	7	9	10	9	14	16	18	25	22	132
Unknown	2	30	51	50	62	61	35	28	13	5	337
Total	17	81	112	107	111	111	96	85	94	74	888

Chi-squared (27 d.f.) = 138.45 (P<0.001)

Chi-squared (18 d.f.) = 40.03 (P<0.01)
(omitting unknown age group)

TABLE 3.8 Analysis of case records file by age and month

Month	1	2	3	4	5	6	7	8	9	10	11	12	TOTAL
Age group													
Adult	37	44	41	25	14	7	14	22	9	13	21	25	272
Yearling	24	13	15	22	11	4	2	3	1	5	25	22	147
Calves	16	19	16	30	7	7	7	4	2	2	10	12	132
Unknown	67	42	33	52	24	11	7	5	3	21	40	32	337
TOTAL	144	118	105	129	56	29	30	34	15	41	96	91	888

Chi-squared (33 d.f.) = 79.40 (P<0.001)
Chi-squared (22 d.f.) = 54.28 (P<0.001)
(omitting unknown age group).

TABLE 3.9 Analysis of case records file by clinical sign and year

Number of incidents:

Year	1977	78	79	80	81	82	83	84	85	86	Total	χ^2	P
Clinical code (Table 3.1)													
A	2	6	10	11	6	5	12	11	21	23	107	45.52	<0.001
B	-	-	-	-	2	-	-	-	2	-	4		
C	1	6	15	10	16	11	10	6	4	1	80	14.26	NS
D	2	2	8	3	2	6	4	4	1	2	34	10.93	NS
E	5	22	42	51	49	51	35	26	27	11	319	22.48	<0.01
F	2	23	37	36	39	38	29	28	18	10	260	15.26	NS
G	-	4	4	3	2	1	-	3	1	3	21	8.93	NS
H	-	2	2	-	1	-	-	-	-	-	5		
I	-	-	-	2	1	-	-	-	-	-	3		
L	1	12	21	11	12	16	10	12	28	27	150	36.10	<0.001
M	3	11	12	6	8	4	11	5	5	7	72	12.48	NS
N	14	50	61	66	62	60	45	41	45	22	466	14.86	NS
P	-	-	-	-	1	-	-	-	1	-	2		
Q	-	3	1	-	-	-	-	-	-	-	4		
R	2	7	11	13	14	7	5	7	6	3	75	8.46	NS
S	-	8	7	8	5	4	2	5	3	1	43	11.52	NS
T	1	8	14	5	7	3	3	1	7	4	53	17.33	<0.05
V	1	-	2	-	-	-	1	1	1	-	6		
X	-	5	14	6	8	13	16	8	3	5	78	17.03	<0.05
Z	1	13	16	9	6	5	4	8	5	9	76	17.28	<0.05
Total cases	17	81	112	107	111	111	96	85	94	74	888		

NS = not significant (P>0.05)

TABLE 3.10 Analysis of case records by clinical sign and month

Number of incidents:

Month	1	2	3	4	5	6	7	8	9	10	11	12	Total	χ^2	P
Clinical code (Table 3.1)															
A	13	9	6	4	6	4	12	20	8	5	9	11	107	118.2	<0.001
B	1	2	–	–	1	–	–	–	–	–	–	–	4		
C	13	15	15	6	4	2	–	2	1	3	12	7	80	12.89	NS
D	3	8	5	3	2	–	–	–	–	1	7	5	34	12.85	NS
E	60	50	39	44	23	10	9	6	1	15	36	26	319	11.75	NS
F	44	48	38	30	12	8	9	4	3	7	31	26	260	16.24	NS
G	1	2	3	6	4	–	1	–	–	1	3	–	21	14.64	NS
H	1	–	–	–	3	–	–	–	–	1	–	–	5		
I	–	–	–	1	–	–	–	2	–	–	–	–	3		
L	25	16	16	26	13	4	6	7	–	8	14	15	150	6.73	NS
M	10	18	11	11	3	3	2	2	1	1	7	3	72	13.75	NS
N	72	62	63	75	28	15	8	9	7	21	58	48	466	11.61	NS
P	–	–	–	–	–	1	–	–	–	–	–	1	2		
Q	–	1	–	1	–	1	–	–	–	–	–	1	4		
R	8	14	11	13	5	3	2	1	1	2	5	10	75	8.01	NS
S	6	9	7	4	4	2	1	–	–	2	2	6	43	9.02	NS
T	7	8	5	7	4	–	–	1	1	5	7	8	53	9.09	NS
V	2	–	1	–	–	–	1	–	–	1	–	1	6		
X	17	11	7	10	5	3	1	1	–	5	7	11	78	7.88	NS
Z	9	7	9	13	11	1	1	5	1	6	9	4	76	17.33	NS
Total cases	144	118	105	129	56	29	30	34	15	41	96	91	888		

NS = not significant (P>0.05)

TABLE 3.11 Summary of sample types used for BHV1 isolation and antigen detection (CVL case records file).

No. incidents:

Sample	Virus isolated	Antigen detected
Nasal swab	285	10
Eye swab	128	3
Lung	59	6
Trachea	42	2
Alimentary tract	18	-
Fetus	12	2
Vagina	2	-
Nervous system	2	-
Blood	3	-
Prepuce	-	-
Miscellaneous	8	-
Total	460	22

Chapter 4

COMPARISON OF FIELD ISOLATES OF BOVID HERPESVIRUS 1 BY DNA FINGERPRINTING

INTRODUCTION

Six field isolates of BHV1, as used for experimental pathogenicity studies (Chapter 5), were examined by the restriction endonuclease fingerprinting technique. The three endonucleases most useful for distinguishing viral genotypes were selected and used to characterize a further 71 British isolates, together with the two live vaccine strains available in Britain and a miscellany of isolates from other countries.

MATERIALS AND METHODS

Kirby phenol

Phenol (A.R. grade - BDH) 500g was melted at 60°C in a water bath, 200ml distilled water and 0.5g 8-hydroxyquinoline added quickly, then (with care) 70g m-cresol.

Restriction digest buffers

The restriction endonucleases were obtained from Bethesda Research Laboratories and the digestion conditions and buffers recommended by the manufacturers were complied with. In each case 100µg/ml nuclease-free bovine serum albumen was added to the buffer prior to incubation.

EcoRI

Tris 50mM, magnesium chloride 10mM, sodium chloride 50mM, dithio-

threitol 1mM, pH adjusted to 7.5. Digestion 4 hours at 37°C.

Hind III

Core buffer as supplied with the enzyme, pH adjusted to 7.4. Digestion 4 hours at 37°C.

Hpa I

Tris 20mM, magnesium chloride 10mM, potassium chloride 20mM, dithiothreitol 1mM, pH adjusted to 7.4 Digestion 1 hour at 37°C.

Bam HI

Tris 20mM, magnesium chloride 7mM, sodium chloride 100mM, 2-mercaptoethanol 2mM, pH adjusted to 8.0. Digestion 4 hours at 37°C.

Pst I

Core buffer as supplied with the enzyme, pH8.0. Digestion 4 hours at 37°C.

Bst EII

Core buffer as supplied with the enzyme, pH8.0. Digestion 1 hour at 60°C.

Stop-sink solution

EDTA	10mM
Bromophenol blue	0.01%
Sucrose	25% w/v

Electrophoresis buffer

Tris	38mM
Sodium di-hydrogen phosphate	28mM
EDTA	1mM

pH to 7.8

Virus preparation

Virus stocks were prepared in primary calf kidney cells as described in Chapter 2. The culture harvest was brought to concentrations of 0.5M sodium chloride, 20mM tris-hydrochloride pH7.4, and 9% w/v polyethylene glycol (molecular weight 6000-8000). After thorough mixing the suspension was held overnight at 4°C then centrifuged for 1 hour at 8000g. The precipitate was resuspended, to approximately one tenth of the original volume, in 20mM tris-hydrochloride buffer pH7.4 then left to stand for 3 to 4 hours to ensure complete dispersion of the pellet.

DNA extraction

An equal volume (typically 10ml) of kirby phenol was added to the concentrated virus suspension, followed by gentle roller mixing for 15 minutes. The emulsion was centrifuged at 1000g for 15 minutes, the aqueous layer decanted off and extracted with a further treatment of kirby phenol. After a second centrifugation the aqueous layer was shaken with 10ml chloroform: n-octanol (9:1) and left to settle. The aqueous layer was retreated with chloroform: n-octanol, then twice with chloroform alone. The aqueous fraction (purified DNA) was dialysed overnight against 10mM tris-hydrochloride pH8.0, 1mM EDTA. The DNA concentration was calculated from the absorbance

at 260nm (50µg/ml corresponded to absorbance of 1.00) and the solution concentrated to 500µg/ml, made 0.02M with sodium chloride, and stored at 4°C.

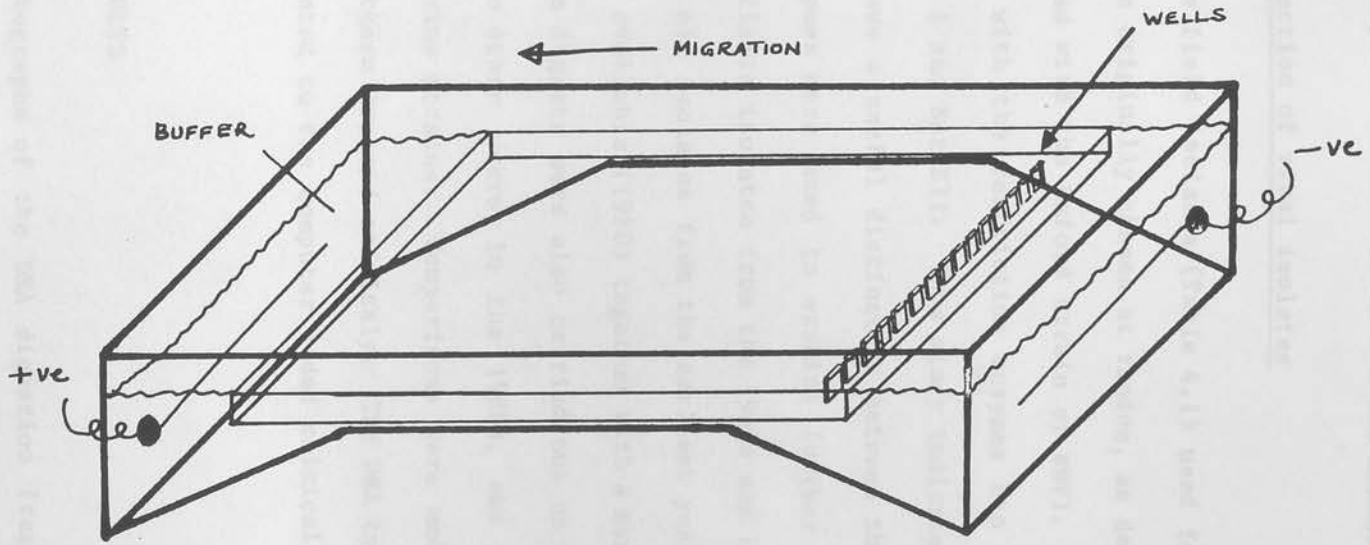
Restriction digests

Equal volumes of 30µl digestion buffer with albumen and DNA solution were mixed. The enzyme (5µl) was added and incubated with gentle mixing under the appropriate time and temperature conditions. Digestion was stopped by the addition of 15µl stop-sink solution.

Electrophoresis

Agarose (Seakem low electroendosmosis - Miles Laboratories) was prepared in 0.5% gels in electrophoresis buffer in a Bethesda Research Laboratories submerged horizontal slab gel tank (Fig 4.1) with a gel depth of 6mm. Aliquots of digestion mixtures (40µl) were pipetted into the wells, together with appropriate molecular weight markers (restriction digests of lambda phage: DNA molecular weight markers I or II, Boehringer-Mannheim). The gel was just covered with buffer and electrophoresis carried out for 16 hours at 30 volts and 125 milliamps. The DNA was stained by the addition of 2mg ethidium bromide to the buffer. After 1 hour the gel was photographed under ultra-violet light in a darkroom, using a Polaroid camera. The relative migration distances were measured on the photographic prints using a precision digital caliper (Jocal, C.E. Johansson Ltd).

The sizes of the digestion fragments were estimated (in kilobase pairs - kbp) with reference to the standard markers by using the computer program "Fragsize" described (and supplied) by Campione Piccardo (1986). This is based on the equations of Southern (1976)



Drawing courtesy of Mrs H White

Fig. 4.1 Electrophoresis tank used for submerged horizontal slab gels to separate restriction digest fragments of viral DNA

which relate molecular size to the reciprocal of the mobility and incorporate a correction for non-linearity.

Selection of viral isolates

Five field isolates (Table 4.1) used for experimental animal studies were originally chosen at random, as described in Chapter 5, and compared with the Oxford strain of BHV1. These six strains were digested with the restriction enzymes Eco RI, Hind III, Hpa I, Bam HI, Pst I and Bst EII. This study indicated that EcoRI, Hind III and Hpa I gave a useful distinction between the main BHV1 genotypes. These enzymes were used to examine further British isolates, namely all available isolates from the 1960s and 1970s, although these were few, and all isolates from the earliest year when an extensive collection was available (1980) together with a more recent year (1984). Restriction digests were also carried out on miscellaneous British isolates from other years in the 1980s, and the currently available live vaccine strains. Comparisons were made with strains from the USA, Northern Ireland and Italy. The DNA type of the British isolates was related to the computer coded clinical signs as described in Chapter 3.

RESULTS

Photographs of the DNA digestion fragments of strains ED1-ED6 are shown in figs 4.2 and 4.3. The calculated fragment sizes based on means of several gels are given in appendices 4 (i-vi) and shown graphically in figs 4.4 - 4.9. Although there were some discrepancies in absolute molecular weights between the results and those of Mayfield and others (1983) it was concluded that strains ED1, ED3,

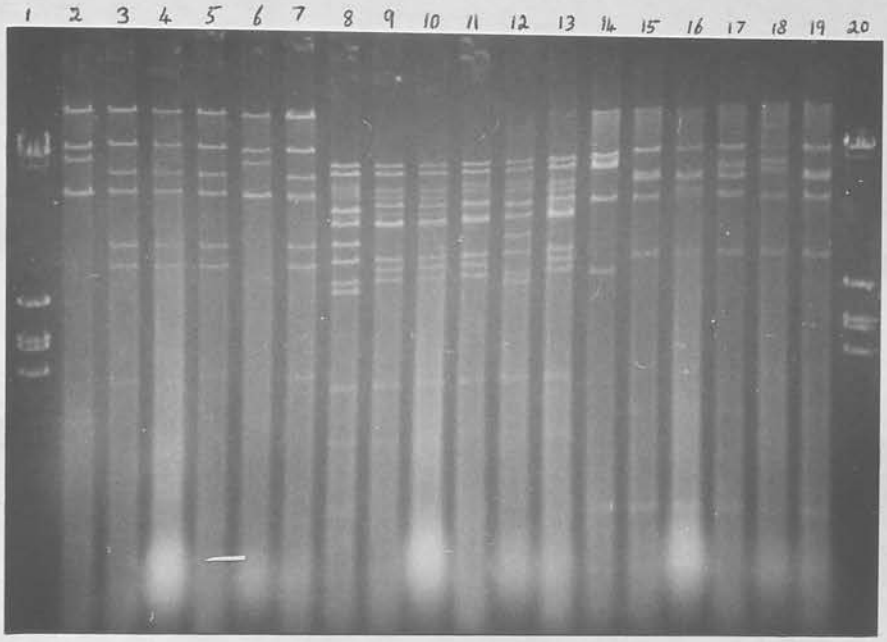


Fig. 4.2 Ethidium bromide stained gel photgraphed under ultra violet light, showing restriction digest fragments of BHV1 strains ED1-ED6 with Hpa I, Hind III and Eco RI

Lanes 1 and 20: molecular weight marker I (Boehringer Mannheim)
Lanes 2-7: Hpa I digests of strains ED6-ED1 respectively
Lanes 8-13: Hind III digests of strains ED6-ED1 respectively
Lanes 14-19: Eco RI digests of strains ED6-ED1 respectively

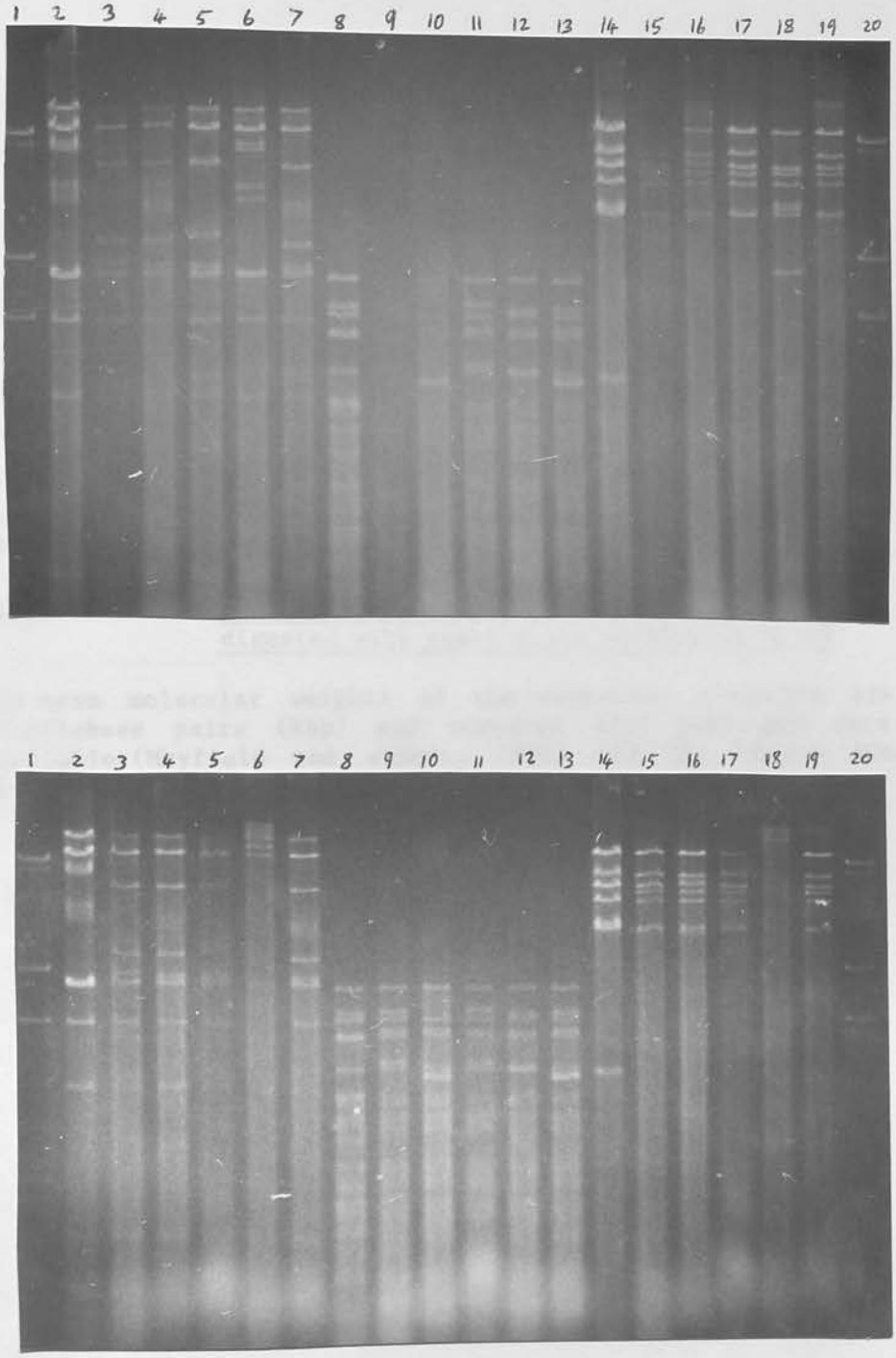


Fig. 4.3 Photographs of two gels which together show the restriction digest fragments of strains ED1-ED6 with Bst EII, Pst I and Bam HI

Lanes 1 and 20: molecular weight marker II (Boehringer Mannheim)
Lanes 2-7: Bst EII digests of strains ED6-ED1 respectively
Lanes 8-13: Pst I digests of strains ED6-ED1 respectively
Lanes 14-19: Bam HI digests of strains ED6-ED1 respectively

Following pages:

Figs. 4.4-4.9 DNA fingerprints for BHV1 strains ED1-ED6 digested with restriction enzymes as shown

The mean molecular weights of the digestion fragments are shown in kilobase pairs (Kbp) and compared with published data where available (Mayfield and others, 1983) for the Cooper (Co.) and K22 strains.

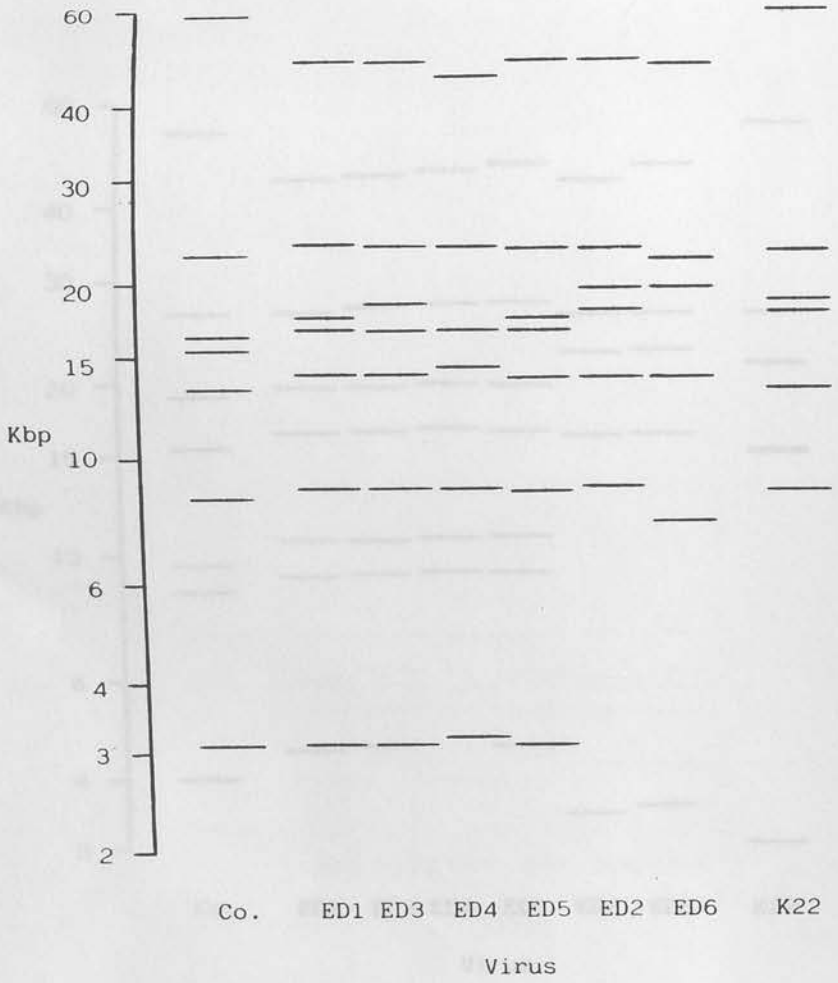


Fig. 4.4 Eco RI digests

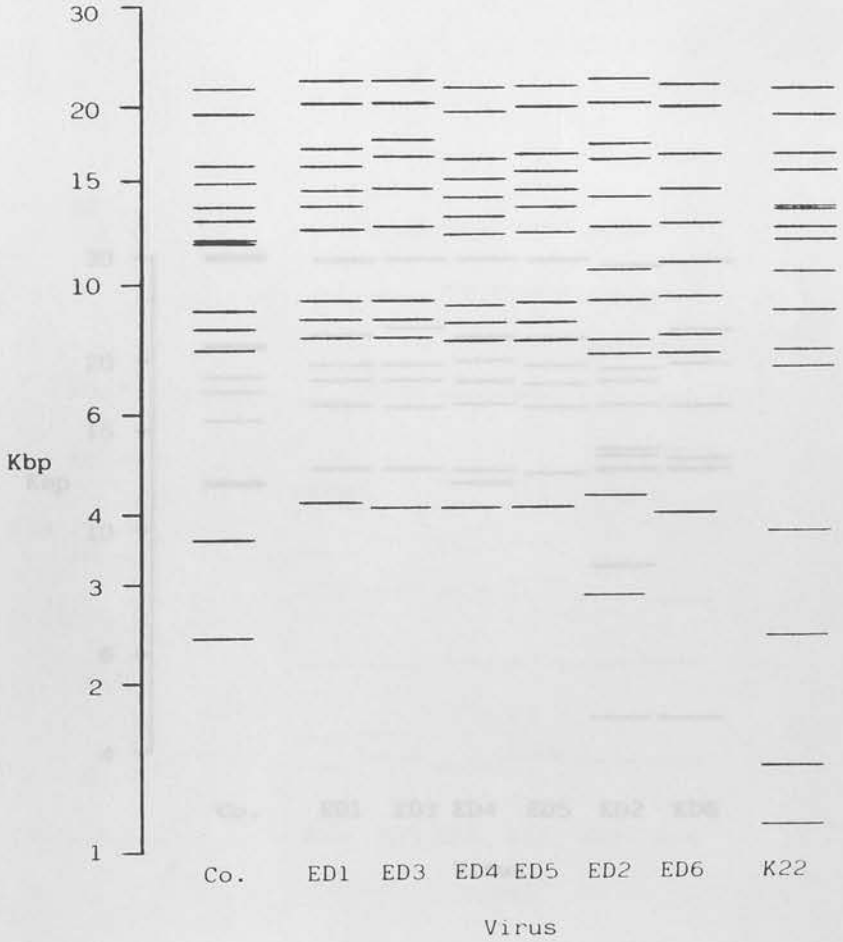


Fig. 4.5 Hind III digests

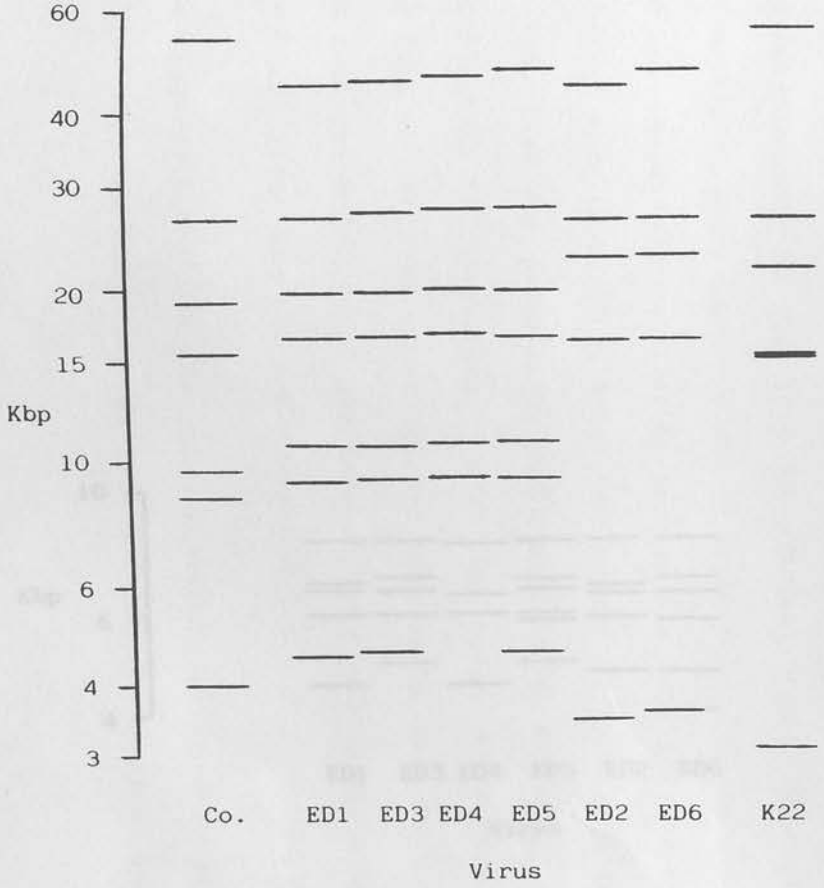


Fig. 4.6 Hpa I digests

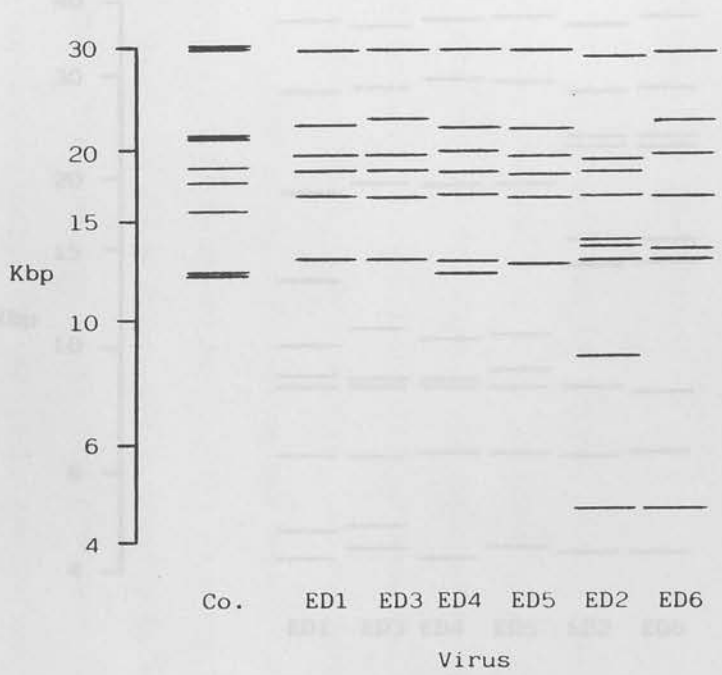


Fig. 4.7 Bam HI digests

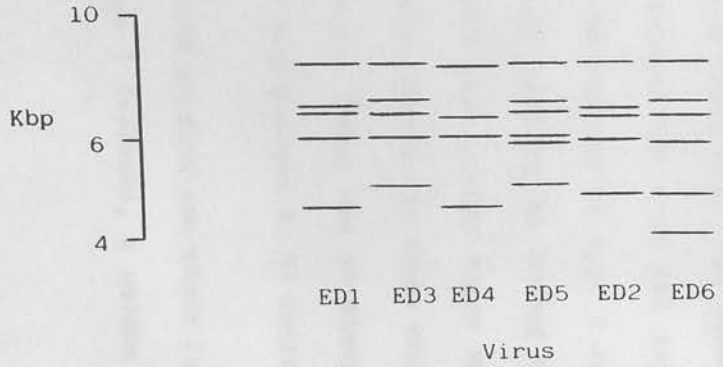


Fig 4.8 Pst I digests

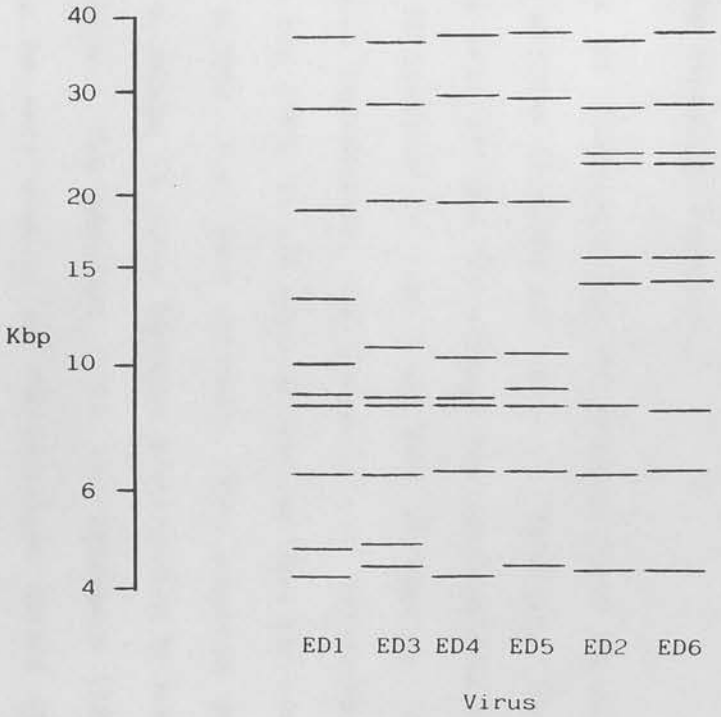


Fig 4.9 Bst EII digests

ED4 and ED5 were of DNA type 1 (Metzler and others, 1985) and ED2 and Oxford (ED6) of type 2b. It was also concluded that the strains were all genetically distinct as shown by minor differences in the mobility of their DNA digestion fragments.

The results of fingerprinting 78 British field isolates of BHV1 (including strains ED1-ED6) are shown in Table 4.2. The four 1960s strains all were of type 2b, whereas the combined data for 1980 and 1984 gave 52 isolates of type 1 and four of type 2b. Assuming the isolates were independent, the probability of obtaining 4/4 type 2 strains in the 1960s if the distribution had been the same as in the 1980s was 0.0001, i.e. very unlikely. This supports the view that there was a change in virus DNA-type distribution between the 1960s and the 1980s. The Aberdeen (IBR) and Aylesbury (IPB) isolates appeared to be very similar to the prototype Oxford (IBR) strain, whereas the Carmarthen (IPB) isolate differed slightly in its fingerprinting with EcoRI and Hind III (Figs. 4.10 and 4.11).

The relationship of DNA type to clinical signs reported in the corresponding outbreaks is shown in Table 4.3. Chi squared analysis did not reveal any association between DNA type and any particular clinical sign ($P>0.10$) although the numbers of type 2 isolates were so low, that any association would need to be strong to be demonstrable. It is interesting to note that ocular signs were recorded for five of the seven type 2 iolates, whereas in cases where mortality was recorded only type 1 was found. There was no discernable geographical trend when the isolates were grouped by VI Centre of origin.

The fingerprints of the live vaccine strains are shown in Figs. 4.10-4.12. Although both resemble type 1 isolates, a unique pattern was

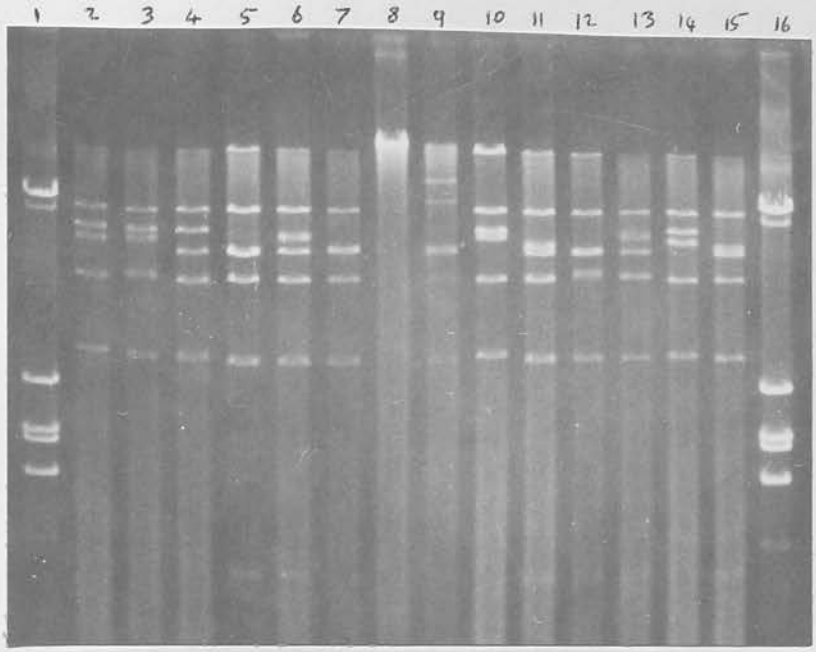


Fig. 4.10 Eco RI digestion fragments of BHV1 strains

Lanes 1 and 16: molecular weight marker I
Virus strains: lane 2 Aberdeen; 3 Aylesbury; 4 Carmarthen;
5 Nasalgen vaccine; 6 Tracherine vaccine;
7 fetal isolate; 8, 9 incomplete digestion;
10 ED6 (Oxford); 11 ED5; 12 ED4; 13 ED3;
14 ED2; 15 ED1

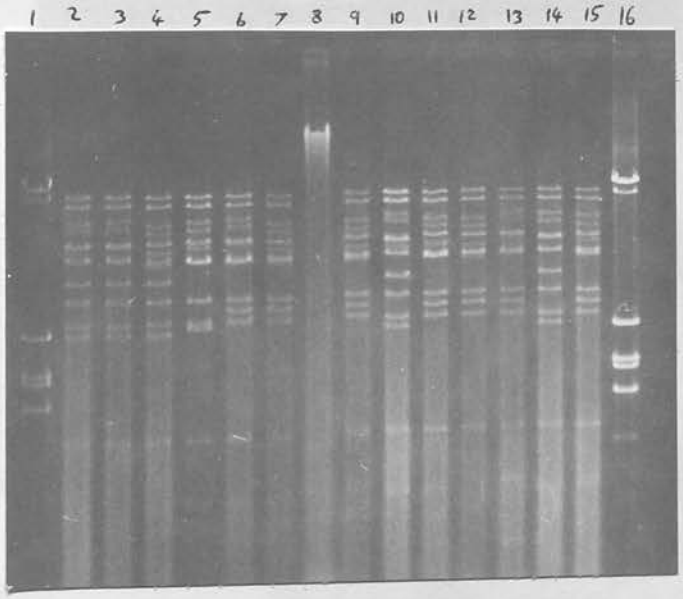


Fig. 4.11 Hind III digestion fragments of BHV1 strains
Lanes as for Fig. 4.10

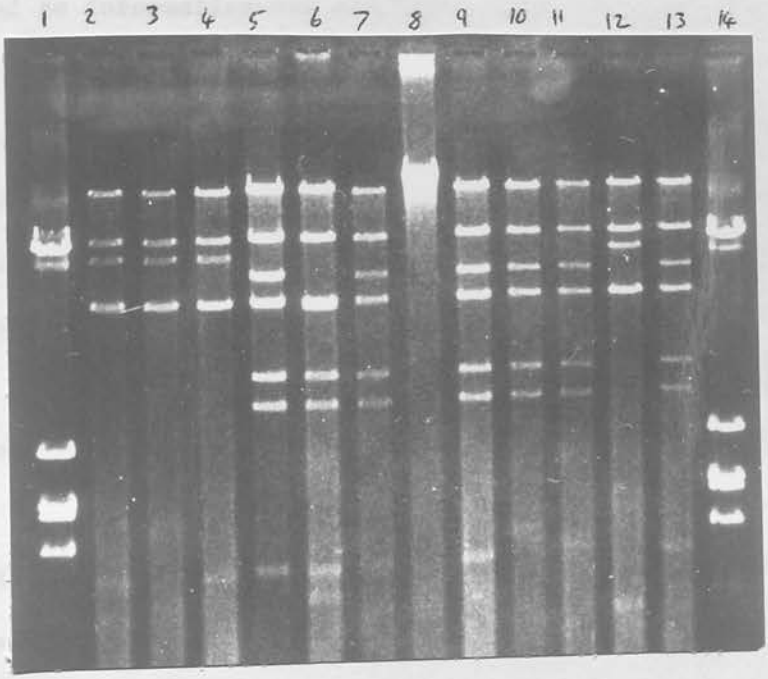


Fig. 4.12 Hpa I digestion fragments of BHV1 strains

Lanes 1 and 14: molecular weight marker I
Virus strains: Lane 2 Aberdeen; 3 Aylesbury; 4 Carmarthen; 5 Nasalgen vaccine, 6 Tracherine vaccine; 7 fetal isolate; 8 incomplete digestion; 9 ED5; 10 ED4; 11 ED3; 12 ED2; 13 ED1

shown by Nasalgen digested with Hind III and by Tracherine with Hpa I. The Tracherine pattern was only once detected in a field isolate, as noted in Table 4.2. This was from a farm where Tracherine had been used, and the isolate possessed the temperature sensitive growth pattern of Tracherine. The Nasalgen pattern has also been identified once in a field isolate (in 1987) from a farm where the cattle had been vaccinated with Nasalgen four days previously. The fingerprints are therefore believed to be specific markers for these vaccine strains.

The American strains of BHV1 had been stored at the CVL since the 1960s and no information was available other than that shown in table 4.4. The IPV strain was believed to be the prototypic K22 strain (Kendrick and others, 1958). The LA, Colorado and Blythe strains, were among the prototypic IBR isolates of the 1950s (Madin and others, 1956; York and others, 1957). The Colorado strain is believed to be synonymous with the Cooper strain mapped by Mayfield and others (1983). The American type 1 viruses all had closely similar fingerprints which corresponded most nearly to strain ED3 among the six British isolates studied in detail. The type 2 strains resembled strain ED2. The four IBR isolates received from Italy had type 1 DNA fingerprints, whereas the five from Northern Ireland were of type 2b (Table 4.4).

CONCLUSIONS

Early British isolates of BHV1 correspond with DNA type 2 along with the prototype American IPV strain. More recent isolates, since 1977, are predominantly of DNA type 1, resembling the prototype IBR strains.

Northern Ireland appears to be in the same situation as Great Britain before 1977 in that only type 2 strains have been identified. This is supported by fingerprinting of more isolates at the Veterinary Research Laboratory, Stormont (McFerran, personal communication, who also reports the absence of virulent IBR in that country). The fact that current British isolates correspond genetically to strains found in the USA in the 1950s and 1960s, as well to strains from other countries, suggests that the current wave of type 1 viruses arose through importation rather than mutation of pre-existing British strains.

TABLE 4.1 - Details of field isolates of BHV1 used for restriction endonuclease typing and for experimental calf inoculations

REF. NO.	VIROLOGY DEPT. NO.	DATE OF ISOLATION	SAMPLE FROM WHICH ISOLATED	SUBMITTING VI CENTRE	CLINICAL SIGNS
ED1	78/4242	28.2.78	nasal swab	Wolver-hampton	Yearlings with respiratory disease following purchase 2 weeks previously
ED2	78/3887	22.2.78	eye swab	Weybridge	Conjunctivitis
ED3	80/9689	30.4.80	trachea	Sutton Bonington	Yearling with dyspnoea. At necropsy, consolidation of apical and cardiac lobes of lung, diphtheritic necrosis of trachea, laryngeal ulceration
ED4	80/10638	12.5.80	nasal swab	Carmarthen	Dullness, anorexia, tachypnoea temperature 103°F, scleral congestion, nostrils inflamed and painful with mucoid necrotic discharge, occasional cough
ED5	80/12514	10.6.80	eye swab	Weybridge	18 month old Friesian male with dullness, fever (105.5°F) and serous nasal and ocular discharges
ED6	Oxford strain (Dawson & others, 1962)	1961	eye swab	Reading	Conjunctivitis, ocular discharge rhinitis, cough in store cattle

TABLE 4.2 - Classification of British BHV1 isolates by DNA type and year of isolation

YEAR	NO. OF ISOLATES			
	TYPE 1	TYPE 2	TRACHERINE	TOTAL EXAMINED
1961	-	1[a]	-	1
1962	-	1[b]	-	1
1969	-	2[c]	-	2
1977	1	-	-	1
1978	4	3	-	7
1980	31	1	-	32
1981	2	-	-	2
1982	-	-	1[d]	1
1984	21	3	-	24
1985	6	-	-	6
1986	1	-	-	1

Notes : a - Oxford IBR strain (Dawson and others, 1962)

b - Aberdeen IBR strain (Darbyshire and Shanks, 1963)

c - Carmarthen IPB strain (Huck and others, 1971) and Aylesbury IPB strain

d - this isolate was from a farm where Tracherine vaccine had been used. It also showed temperature sensitive growth characteristics

TABLE 4.3 - Classification of 74 post-1977 isolates of BHV1 by DNA type and clinical signs

CLINICAL CODES (AS IN TABLE 3.1)	DNA TYPE			TOTAL
	1	2	Tr	
A	2	-	-	2
B	2	-	-	2
C	6	-	-	6
D	4	-	-	4
E	31	5	-	36
F	27	1	-	28
G	5	-	-	5
H	-	-	-	-
I	-	-	-	-
L	8	-	1	9
M	3	1	-	4
N	45	3	-	48
P	-	-	-	-
Q	-	-	-	-
R	7	1	-	8
S	6	1	-	7
T	3	-	-	3
V	2	-	-	2
X	2	-	-	2
Z	14	-	-	14
TOTAL NO. OF ISOLATES	66	7	1	74

Tr = Tracherine vaccine type

TABLE 4.4 - Restriction endonuclease typing of overseas strains of BHV1 (based on digests with EcoRI, Hind III, HpaI and Bam HI)

COUNTRY OF ORIGIN	STRAIN REFERENCE	DNA TYPE
USA	Colorado	1
	IPV	2
	Ledi IV	2
	Salinas VII	2
	Blythe VII	1
	Conjunct	1
	Kleck IX	1
	McDougall VII	1
	LA III	1
	Reno	1
	Palmer	1
	Nanny V	1
ITALY	V77 (IBR)	1
	80 (IBR)	1
	82 (IBR)	1
	13[a]	1[a]
NORTHERN IRELAND	VC 64[b]-100 (IBR)	2
	VC 74-235 (subclinical infection in a bull)	2
	VC 74-366 (calf pneumonia)	2
	VC 82-569 (IBR)	2
	EC 84-11/73 (subclinical infection in a herd)	2

Notes : (a) this strain was of uncertain origin having been found in a deep freeze. Plaque cloning revealed a mixture of two distinct genotypes, one of type 1 and one with additional bands.

(b) the first two digits of the Irish strains represent the year of original isolation

Chapter 5

CLINICAL AND SEROLOGICAL RESPONSES OF EXPERIMENTALLY INFECTED CALVES

INTRODUCTION

In order to establish a satisfactory system of clinical evaluation, together with appropriate sampling regimens, a pilot experiment was carried out by experimentally infecting calves with different strains of BHV1. Five recent isolates were selected at random and compared with the UK prototype strain, "Oxford". Fortuitously, when restriction endonuclease mapping was subsequently applied to the 6 strains, it was found that one of the 5 recent isolates had a genotype similar to the Oxford strain (type 2), whereas the other 4 corresponded to DNA type 1. This aspect has been fully described in chapter 4. To confirm the trends observed in the pilot experiment, two of the strains, both first isolated in 1978, and corresponding to the two DNA types, were selected for a more detailed assessment of the clinical, virological and serological responses of calves to experimental inoculation.

MATERIALS AND METHODS

Experiment 1

In the pilot study, each of the six selected BHV1 strains was inoculated into three calves. For logistical reasons it was necessary to subdivide the experiment into three replicate blocks (A, B and C), in each of which one calf was allocated per viral strain. Additionally, one or two calves per block were sham inoculated with the medium harvested from non-infected cell cultures. These calves served as

controls both for spontaneously arising intercurrent diseases, and to monitor the efficacy of disinfectant procedures for personnel passing between the calf pens. In blocks A and C the calves were housed in individual loose boxes, divided to ceiling height by a solid wall, and with solid doors opening onto a common service passage containing hypochlorite dips between each pen. The pens were individually fan ventilated. In block B the calves were housed in individual self-contained calf hutches, naturally ventilated through weld mesh fronts and sides, and standing in a concrete yard with 1 to 1.5 metres separation between the hutches. All staff attending the calves were obliged, for the period of the experiments, to wear waterproof clothing and to disinfect between each calf pen.

The intention was to use calves which were, at least within blocks, of a uniform type, age and size, although in practice this was partly confounded by what was available at the time. In blocks A and B the calves were either dairy-type or beef crosses from this type, and were of mixed sexes (Table 5.1). They were purchased at the usual market age (approximately 1-2 weeks) and were experimentally inoculated 2-4 weeks later. Better uniformity was achieved for block C where the calves were all Friesian-type and male. The calves were allocated to treatments (i.e. viral strains) by random number selection.

Virus stocks grown in primary calf kidney cells were diluted in cell culture medium to provide an inoculum of each strain containing 3.5×10^7 $TCID_{50}$ per ml. Each calf was inoculated with 5 ml of this preparation by intranasal installation, plus a further 0.5 ml dropped onto the surface of the left eye. The nasal and ocular inoculations were repeated at 2 and 4 hours after the initial dose. Each calf thus received a total viral dose of 5×10^8 $TCID_{50}$ intranasally

plus 5 x 10^7 $TCID_{50}$ supraconjunctivally. The five recent isolates of the virus had been passaged four times in cell culture from the original field sample, whilst the Oxford strain was used at the 6th passage level, having been grown from liophylized 5th passage material. Details of the six viral strains used (referred to as ED1 to ED6) were given in chapter 4 (Table 4.1).

Experiment 2

For the second phase of the study the opportunity was taken to use Friesian calves from the CVL Ripley herd, which was being dispersed in 1983 as part of government economies. This herd had been built up over a number of years and maintained free of specific pathogens, including BHV1 and BVDV. To offset the advantage of having these calves from a single source and of known history, it was necessary to accept a wider age range (2½ to 5 months on the date of inoculation) and mixed sexes. They were divided into age-matched pairs of calves, then randomly allocated, within pairs, to two treatment groups (Table 5.2). The six calves for each group were housed together in a large loose box, separated by solid walls and an empty box from the other group. Strict disinfection procedures between groups were maintained, as in experiment 1. The viral strains selected for experiment 2 were ED1 and ED2, both at the third cell culture passage level. The inocula were prepared as before, but a lower dose was given than in experiment 1. Each of four calves in each treatment group was inoculated intranasally with 10^7 $TCID_{50}$ virus in a single dose of 1 ml, plus $10^6 TCID_{50}$ supraconjunctivally in the left eye, in a volume of 0.1 ml. The remaining two calves per group served as in-contact controls.

Following recovery from the acute infection and the conclusion of the initial monitoring period, the three castrated male calves from this experiment were separated from their groups and were inoculated, 56 days after the BHV1 inoculation, with 8 mg dexamethasone intravenously, followed, on each of the subsequent four days, by the same dose intramuscularly. The pattern of recrudescent virus shedding was monitored by the daily collection of nasal and preputial swabs. On day 88 after the original BHV1 inoculation, two of the remaining female calves from each group (numbers 9378, 9406, 9407 and 9408) were challenge inoculated with the opposite strain of virus (ED2 or ED1 respectively) but at the same dose and routes as the original inoculation.

Experiment 3

The final calf study was carried out as a minor addition, to test the hypothesis, suggested by the results of experiment 1, that there was a difference in virulence between two strains (ED1 and ED5) of apparently similar genotypes. The six calves selected were, for reasons of economy and availability, male Jerseys, of uniform age. The inoculation was carried out when the calves were 2 months old, using the same dose of virus and routes of inoculation as for experiment 2. The housing was similar (but not identical) to experiment 2, and both experiments 2 and 3 were done in the same month (July) in successive years.

Sampling procedure

In all the calf experiments, a standard procedure was adopted for sampling and clinical monitoring. Cotton wool swabs (Exogen Ltd.,

Clydebank, Scotland) were inserted ventromedially through the nostril to the full extent (approximately 11 cm) of their wooden sticks. One nasal swab was taken per day from each calf and was inserted into both nostrils in turn. Separate swabs were used to sample the lachrymal secretions of the left and right eyes, by gently everting the lower eyelid. The swabs were broken off into 3 ml PBSL in screw capped vials, and processed for antigen detection and virus isolation/titration as described in chapter 6. All the swabs collected on one of the sampling days (experiment 1B, day 8) were weighed before and after sampling, to determine the amount of mucus collected. Swabs were taken immediately prior to viral inoculation, then daily throughout the monitoring period, except for experiment 3 where nasal swabs only were collected, on days 4, 7, 9 and 11 after inoculation.

Clotted blood samples were collected by evacuated blood tubes (Vacutainer, Beckton Dickinson) from the jugular veins of the calves at varying intervals, as indicated in the serology results section. After overnight storage, the sera were separated from the blood clots and stored at -20°C to await serological testing. The serological test procedures (virus neutralization and ELISA) are described in chapter 2.

In block C of experiment 1 and in experiment 2, samples of nasal mucus for serology were collected by inserting a tampon (Lil-lets mini tampons, Lilia White Ltd., Birmingham) into one nostril of the calf, pulling it out 20-30 minutes later, and squeezing out the mucus by compression in the barrel of a 50 ml syringe.

Clinical assessment

All the viral and sham inocula were coded by an assistant and sup-

plied to the author in identical bottles so that I did not know, during the monitoring period, which calf had received which inoculum. Each calf was fully examined clinically, at the same time daily, for one week before the inoculation and for 11-15 days after inoculation. The longer period was used in experiment 2 where disease in the in-contact calves ran a time course some days later than the inoculated calves. Rectal temperature was measured with an electronic, digital thermometer (Model GC20, Edale Instruments Ltd., Cambridge) accurate to 0.1°C. The clinical findings were recorded on a daily record sheet without reference (other than memory) to the previous days' results. A specific score, graded according to severity, was allotted for each of a list of signs for each calf on each day, as shown in table 5.3. The list of signs was constructed on the basis of the clinical findings in the initial experiments. The absence of other signs from the list, for example mouth lesions, does not imply a less than complete clinical examination but merely that no clinical score was allotted for that particular sign in any of the calves during the course of the experiments. In assessing the scores for apathy and anorexia, a knowledge of the individual calves' general behaviour, gained during the pre-inoculation monitoring, was of considerable value. Due regard was also given to comments by the animal attendant, particularly in respect of feeding behaviour and appetite. Throughout this series of experiments, all the clinical assessments were made by the same veterinary surgeon.

At the end of the intensive monitoring period for each experiment, the raw clinical scoring data was transformed to give a numerical assessment of the clinical severity of the disease in each calf. A number of methods were tried in order to establish the most satisfac-

tory assessment, as elaborated in the discussion. The selected method used for the definitive analysis of the data is described here.

The score for each clinical sign was multiplied by a weighting factor (shown in Table 5.3). Although arbitrary, this was an attempt to apportion greater significance to signs such as apathy and anorexia, which were considered to indicate the calf's general well-being, as opposed to more specific signs such as nasal discharge. Because only one eye was inoculated, the ocular signs for the two eyes were assessed separately and given a weighting of 1. The combined left and right eye scores thus had a weight of 2, the same as the other specific signs. The respiratory rates of the calves were rarely elevated, so that the only objectively quantitative measure of the calves' clinical response was the rectal temperature. For this reason, and that it was also considered an important indicator of the calves' general well-being, it was given a considerable weighting, a score of 1 being allotted for every 0.1°C elevation above 39.0°C. The weighted post-inoculation scores for each calf were totalled to give a cumulative score for each clinical sign. Any scores recorded for the same sign during the week of pre-inoculation monitoring were also totalled, then adjusted to give an estimate of an 11 day or 15 day cumulative pre-inoculation score, as appropriate to the period used for calculation of the post-inoculation scores in the particular experiment. The adjusted pre-inoculation score for each clinical sign was then subtracted from the post-inoculation value for that sign. Expressed algebraically, this calculation is:-

$$S = \left[\sum_{m}' D - \frac{(\sum_{0}^{-7} P)m}{7} \right] W$$

Where

S = calculated score for any particular clinical sign for one calf

D = daily score recorded at time of examination in the post-inoculation period

P = daily score recorded at time of examination on days 7-0 pre-inoculation.

m = post-inoculation monitoring period in days

W = arbitrary weighting for the particular sign

The total value of all the clinical signs, ΣS, was used as an overall assessment of the severity of the disease experienced by the individual calf. The temporal progression of the disease was also assessed, by calculating a daily total score (not cumulative) for all the clinical signs in each calf.

Statistical analysis

Experiment 1 was analysed as a 2-way randomized block design in which the six viral strains represented treatments, and the three replicate trials represented blocks. Further analysis was carried out by comparison of two treatment groups representing the two main DNA types. Experiments 2 and 3 were analysed as comparisons of two treatments, using methods for paired comparisons where appropriate.

The cumulative clinical scores for individual calves were analysed by both parametric (analysis of variance and Student's t test) and non-parametric methods (Friedmann's test for randomized blocks, Wilcoxon's rank-sign test for paired comparisons, Mann-Whitney U test for comparing two treatments, and Kruskal-Wallis test for multiple comparisons). The simpler analyses were done manually following standard procedures (Snedecor and Cochran, 1967; Hollander and Wolfe,

1973; Neave, 1981). For the more complex manipulations (such as the Kruskal-Wallis test) the Minitab statistical package, available on the CVL Prime computer, was used.

As explained in the results, the clinical score for one calf (virus ED2, experiment 1A) was considered invalid, and in order to produce a balanced table for the analysis of variance (anovar) an estimated value was calculated by the method of Yates (1933) using the formula:-

$$X = \frac{t.T + b.B - S}{(t-1)(b-1)}$$

where t = number of treatments (virus strains)

T = sum of observations in the treatment with the missing value

b = number of blocks

B = sum of observations for treatments in the block with the missing value

S = sum of all the observations

the total degrees of freedom used = t.b-2

the mean square for treatments was adjusted as described by Snedecor and Cochran (1967) by subtracting from it the value:-

$$\frac{[B-(t-1)X]^2}{t(t-1)^2}$$

In order to compare viral shedding between treatments, a cumulative value for each calf was calculated by summing the $\log_{10}$ of the virus titres in the daily swab extraction media. The data included a high proportion of negative results in which no viral growth was detected from undiluted samples (recorded as '-') or from samples diluted 1/10 (recorded as <1, the undiluted test being unreadable). Both these results were assigned an arbitrary $\log_{10}$ value of 0 for the analyses, for which non-parametric methods based on rank were used as described above.

For the comparison of antibody responses, as measured in OD units by

the ELISA, the data from experiment 1 were analysed for the day on which the response had peaked for the majority of calves. Analysis of variance and Student's t test were used. The missing datum formula (as above) was used for the seropositive calf where necessary to construct balanced tables. An additional two way anovar was done for experiment 1, comparing daily OD values between calves within each block. The ELISA responses for the post inoculation sampling period were compared for the two DNA types by a two sample t test using a pooled variance estimate. To determine the first samples after inoculation in which a rise could be detected in each class of antibody, Student's t test was applied as a paired comparison between OD values on the day of test and those on the day of inoculation (day 0).

Experiment 2 ELISA results were analysed as a paired comparison, using Student's t and Wilcoxon's rank-sign tests on the daily pair differences for the period of the main response (days 11-56 for IgG1 and IgG2, days 11-21 for IgM). Student's t was also used, as in experiment 1, to determine the first sample after inoculation in which antibodies of each class were significantly elevated above day 0. Similar methods were used to analyse the secretory antibody responses in the nasal mucus.

RESULTS

Clinical scores

One calf in experiment 1 (inoculated with strain ED2) failed to develop more than transitory and slight clinical signs of disease, although a successful inoculation was confirmed by the isolation of virus from swabs. In retrospect this calf was found to be seropositive to BHV1 prior to the inoculation. Following this experience the pre-inoculation screening procedures for the calves were tightened up.

With the exception of the above-mentioned calf and the control (sham-inoculated calves in experiment 1, all the calves developed a clinically apparent disease following exposure to the virus. The qualitative features of the disease were similar for all six viral strains. There was typically a high fever beginning on day 2 or 3 after inoculation and lasting for approximately 5 days. A severe conjunctivitis and lachrymal discharge (Fig. 5.1) was evident from about day 2 to day 10 in the left (inoculated) eye, with a milder, more variable response in the right eye. A characteristic feature of the conjunctivitis was the presence of white pustular lesions in both the bulbar and palpebral parts of the conjunctival membrane (Fig. 5.2). Nasal discharge and rhinitis characterized by necrotic ulcerative lesions visible through the external nares, developed slightly later, from about day 5, and were resolving usually by day 10, but with some exceptions noted below. The nasal and ocular discharges were initially clear and watery, but soon showed white flecks and within two days were usually mucopurulent (Fig. 5.3). Coughing was not a consistent finding; when present it was mild and infrequent and generally only provoked when the calves were disturbed. In some calves the upper respiratory changes were severe enough to induce very noisy respirations, akin to snoring, but these were not generally classed as dyspnoea. Only a few calves were judged to be genuinely dyspnoeic and this was transitory, of only one or two days duration. Inappetance and apathy, usually around 5-7 days after inoculation, were inconstant findings and were only observed with some of the viral strains.

The signs of disease had resolved in the majority of the calves by 12 days after inoculation. A few calves had residual slight nasal

Fig. 5.1 Experimentally infected calf showing nasal discharge, ocular discharge and conjunctivitis

Fig. 5.2 Conjunctival injection and pustule formation in an an experimentally infected calf

Fig. 5.3 Mucopurulent nasal discharge in an experimentally infected calf

and/or ocular discharges for a further 2-3 days. One calf in experiment 1B, and one in experiment 1C, developed persistent mucopurulent nasal discharges which were considered on clinical grounds to be due to secondary bacterial infection rather than directly caused by the virus itself. The total clinical scores calculated for experiment 1 are shown in table 5.4. The detailed scores by clinical sign and by days after inoculation are recorded in appendix 5 (i) and (ii). The two-way statistical analysis of the data is shown in table 5.5. The score for the seropositive calf was replaced by an estimated value of 65 for the analysis of variance. The ranking for the Friedmann test was not affected whether the actual or the estimated score was used. Only the Friedmann test gave a significant effect due to virus strains. However, the use of data (either real or estimated) from the seropositive calf, to produce a balanced table, obliges a cautious interpretation of the significance test. A Kruskal-Wallis test on the scores in table 5.4 indicated a significant effect of viral strain if the value of 29 at (a) was included ($H = 12.19$, $P<0.05$) or replaced by the estimated value ($H = 11.84$, $P<0.05$) but not if it was omitted altogether ($H = 10.95$, $P>0.05$).

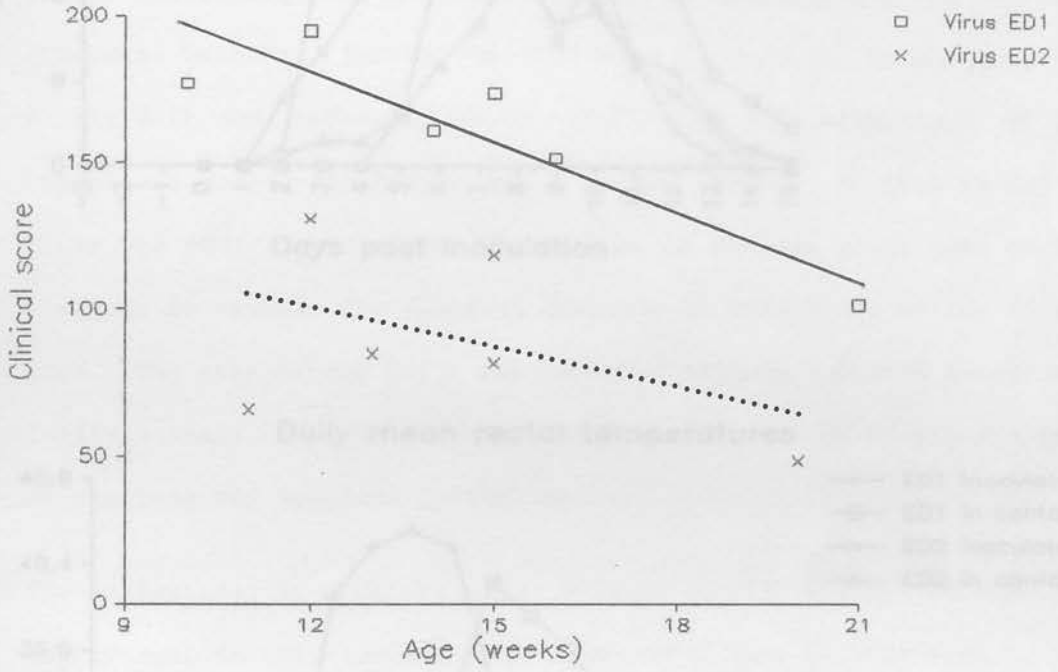
As there was no evidence of significant variation due to blocks, the data were re-analysed as a simple comparison between two treatments, corresponding to the two types of viral DNA identified by restriction endonuclease analysis. The results are shown in table 5.6 and indicate that strains of DNA type 1 produce a significantly more severe clinical response (as assessed by the score) than strains of DNA type 2 given at the same dose by the same route.

The clinical responses following primary inoculation of the calves in experiment 2 are summarised in table 5.7. For every pair of

calves, virus strain ED1 produced a higher clinical score than ED2. The difference between the strains was statistically significant at the 1% level using Student's t test. The finding was confirmed by non-parametric tests which gave significance at the 5% level. The major contributor to the score differences was the rectal temperature (higher fever, rather than longer duration) with increased severity also noted for inappetance (no scores with ED2), rhinitis, nasal discharge, conjunctivitis and ocular discharge. The in-contact calves showed disease of character and severity similar to the inoculated calves for each virus, except that both calves in the oldest pair had a rather lower clinical score than the younger calves infected with the same virus strains. There was a significant correlation between clinical score and age of calf (Fig. 5.4) with strain ED1 ($r=-0.90$, $p<0.05$) but not with strain ED2 ($r=-0.46$, $p>0.05$). No allowance has been made, in fitting the regression lines to fig. 5.4, for any confounding effects of in-contact versus inoculated calves within each strain.

The progression of clinical signs with time is shown in fig. 5.5. The curves for the in-contact calves are displaced approximately three days to the right compared with the inoculated animals.

No clinical signs were detected when the three male calves were given dexamethasone. In the four females which were challenge inoculated with virus of the opposite DNA type, no clinically significant signs were recorded. Small scores were recorded on single days for ocular or nasal discharge or for pyrexia (particularly with ED1 as challenge virus), but none of the scores exceeded values that might be encountered from time to time in "healthy" animals (Table 5.8). Differences



		ED1	ED2
Linear regression parameters:		-7.7	-4.4
	Intercept	272.6	152.1
Significance of correlation:		<0.05	>0.05

Fig. 5.4 Relationship of clinical score to age in calves infected with BHV1 strains ED1 or ED2 (experiment 2)

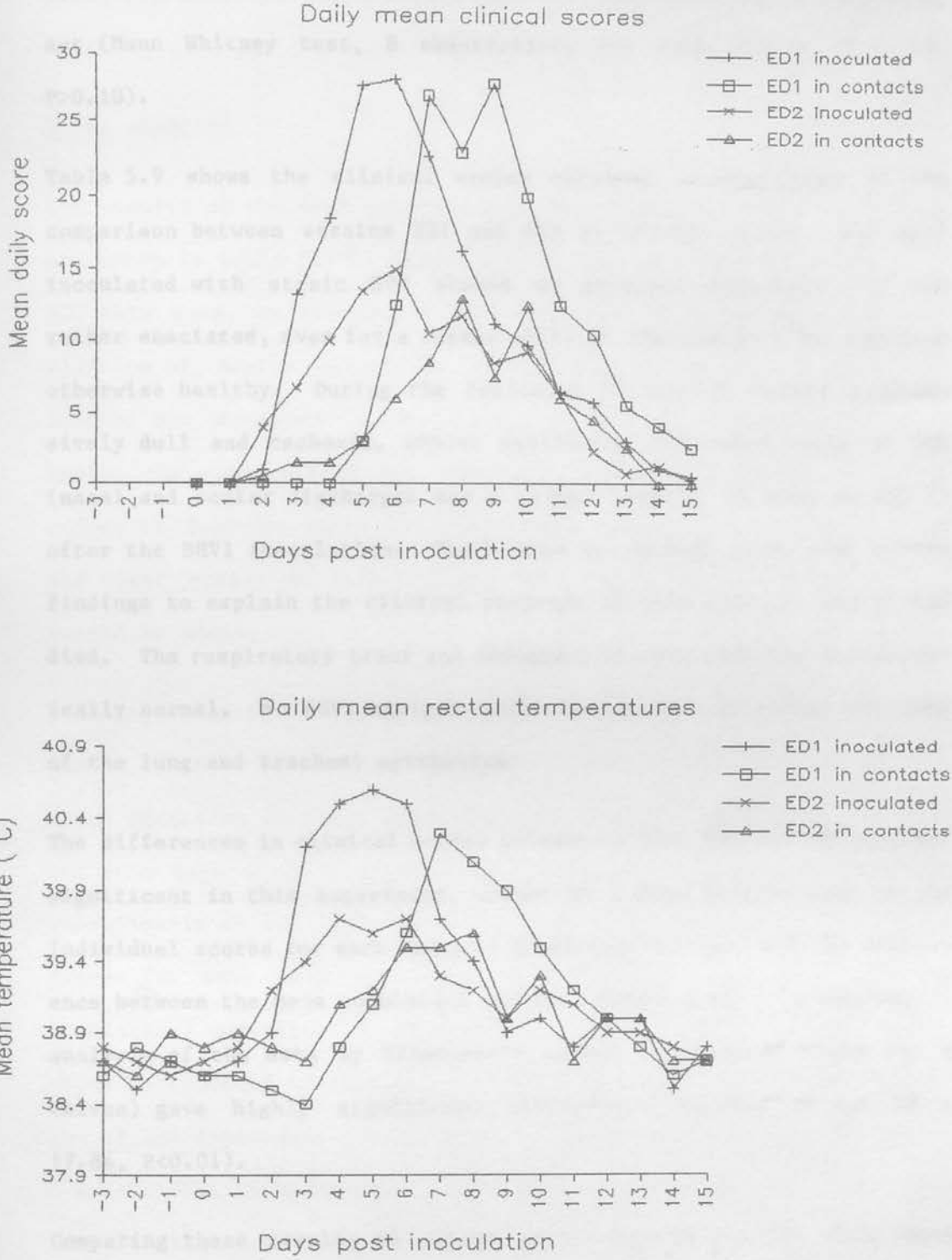


Fig. 5.5 Clinical responses of calves inoculated or in-contact with BHV1 strains ED1 or ED2 (experiment 2)

attributable to the viral strain used for challenge were not significant (Mann Whitney test, 8 observations for each strain, U = 18, P>0.10).

Table 5.9 shows the clinical scores obtained in experiment 3, the comparison between strains ED1 and ED5 in Jersey calves. One calf inoculated with strain ED1 showed an atypical response. It was rather emaciated, even for a Jersey calf, at inoculation, but appeared otherwise healthy. During the following 12 days it became progressively dull and cachexic, whilst exhibiting only mild signs of IBR (nasal and ocular discharges and a slight fever). It died on day 13 after the BHV1 inoculation. There were no obvious gross post mortem findings to explain the clinical response of this calf, or why it had died. The respiratory tract and abdominal viscera appeared macroscopically normal. No BHV1 antigen could be detected in frozen sections of the lung and tracheal epithelium.

The differences in clinical scores between strains ED1 and ED5 were not significant in this experiment, either by a Mann Whitney test on the individual scores for each sign, or by Student's t test for the difference between the mean cumulative scores (Table 5.9). A two-way analysis of the data by Friedmann's method (7 clinical signs vs. 6 calves) gave highly significant differences between calves (M = 17.86, P<0.01).

Comparing these results in Jersey calves for strain ED1 with those obtained earlier in Friesians with the same inoculum (Table 5.7) the clinical responses in experiment 3 were significantly lower (Mann Whitney test, $n_1 = 3$, $n_2 = 6$, U = 1, P = 0.05). Although the two experiments were carried out a year apart, the climate and housing

were very similar, and the only obvious difference was the breed of calf.

Virus shedding

The results of the swab weighing exercise, before and after sampling, are shown in table 5.10. After elution into the extraction medium (3 ml) this gave, on average, a 1/21 dilution of nasal mucus and 1/75 dilution of ocular secretion. The virus titres in the following account are expressed in $\log_{10}TCID_{50}$ per 90µl of extraction media. To express them as estimated $\log_{10}$ titres per ml of mucus, values of 2.37 (= $\log_{10}$ 233) for nasal mucus, 3.05 (= $\log_{10}$ 1111) for right ocular or 2.82 (=$\log_{10}$ 667) for left ocular secretions, should be added.

Details of the daily virus titres from experiment 1 are given in appendix 5(iii). Measurable titres of virus were present in the nasal secretion 24 hours after the inoculation. Peak virus shedding was from days 2 to 5 after inoculation. Virus was detected only sporadically at low titre from day 8 onwards. The cumulative virus titres for each calf are shown in table 5.11, together with the statistical analysis. A significant effect ($P<0.05$) on cumulative virus titre could be attributed to viral strains, whilst the comparison of DNA types gave a very highly significant difference ($P<0.001$). A Kruskal-Wallis analysis of the 198 observations for daily virus titre (Table 5.12) showed significant variation due to DNA type ($P<0.01$) and day of sampling ($P<0.001$), but the differences between strains were not significant. If however the analysis was restricted to samples collected on days one to seven after inoculation, which was the main period of virus shedding and thus excluded a large

number of negative results, the differences between strains were highly significant ($P<0.01$), as were those due to DNA type ($P<0.001$) and day ($P<0.001$). Thus differences in virus excretion between treatments were mainly apparent in the first week after inoculation.

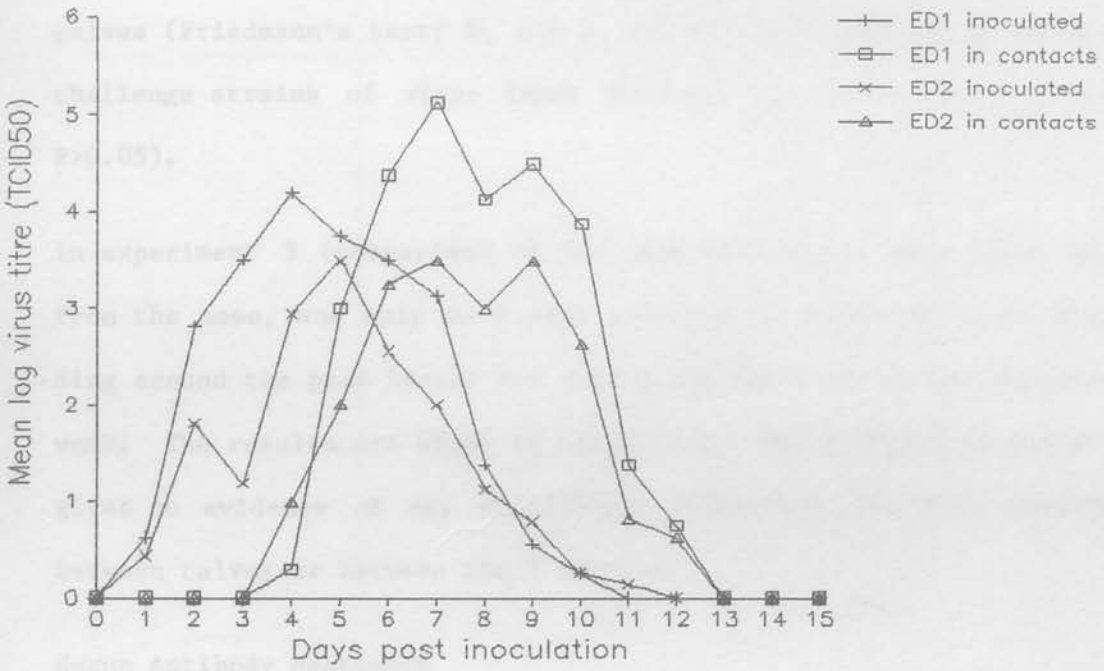
Although overall in experiment 1 there was a significant correlation between the cumulative clinical scores and titres of virus excreted ($r = 0.597$, $P<0.01$), the relationship did not hold within DNA types (type 1, $r = 0.369$, $P>0.05$; type 2, $r = -0.662$, $P>0.05$).

The general pattern of virus shedding in the lachrymal secretions of the left (inoculated) eyes of the calves was similar to that found in the nasal secretion. However, the titres measured were rather variable from day to day, presumably due to the small and variable sample size picked up on the ocular swabs (Table 5.10). Virus recovery from the right (uninoculated) eyes was intermittent and generally at low titres. The detailed results for the eye swabs from experiment 1 are recorded in appendix 5 (iv and v). The results are summarized, together with the statistical analysis, in table 5.13. As with the nasal swabs, a cumulative virus titre was calculated for the Friedmann analysis. No significant effects attributable to blocks or to viral strains were shown. A significant effect of viral strains ($P<0.05$) was demonstrated by the Kruskal-Wallis test on the daily values for virus titre in the left eye samples, but not the right eye. In the Mann Whitney comparison of cumulative titres according to viral DNA type, calves inoculated with type 1 strains shed significantly more virus in their left ocular secretions ($P<0.001$) than those infected with type 2 strains. The differences for the uninoculated (right) eyes were not significant. The virus titration results for the nasal swab samples in experiment 2 are given in table 5.14. The temporal

pattern of virus excretion is illustrated graphically in fig. 5.6. This may be compared with the curves for clinical scores in fig. 5.5. As with the clinical signs, virus excretion by the in-contact calves mimicked that from the inoculated animals with a 3 day time-lag. There was no significant difference in cumulative titre between inoculated and in-contact animals (Mann Whitney, $n_1 = 4$, $n_2 = 8$, $U = 11$, $P>0.10$). For each of the six pairs, the calf infected with strain ED1 had a higher cumulative virus titre than the ED2 infected calf. The differences were significant ($P<0.05$) by both Mann Whitney and Wilcoxon procedures. The Mann Whitney test failed to show a significant difference in virus shedding between strains when the 180 daily titrations were analysed. However when this data was analysed for each pair, the Wilcoxon signed rank test showed significant differences ($P<0.05$) for two pairs (nos. 3 and 4).

Details of the ocular swab titrations in experiment 2 are given in appendix 5 (vi). The calculated cumulative titres and the statistical analysis are in table 5.15. The left eyes of the in-contact calves received no different treatment from any of the right eyes and they were therefore analysed together as a non-inoculated group. As with the nasal swabs, there was a tendency for ED1 infected calves to shed higher titres than the ED2 group. This difference was statistically significant ($P<0.05$) in the non-inoculated eyes using the Wilcoxon signed rank test for a paired comparison, but not by the Mann Whitney method for comparing two treatments.

Virus recovery from the three male calves in experiment 2 following dexamethasone treatment is shown in table 5.16. These samples were screened at 1/2 and 1/10 dilutions of the extraction medium, but the end-point titres of virus growth were not determined. Virus was



Values plotted are the mean log titre per 90 µl swab extraction medium

Fig. 5.6 Pattern of virus excretion in nasal mucus of calves inoculated or in-contact with BHV1 strains ED1 or ED2 (Experiment 2)

detected in all 3 calves during the week after dexamethasone treatment, from both nasal and preputial sampling sites.

The pattern of virus shedding in the four calves following challenge with the opposite strain of BHV1 was similar in all four calves as shown in table 5.17. Only the nasal swabs gave sufficient data for meaningful analysis which showed no significant differences between calves (Friedmann's test; k, n = 4, 11; M = 5.97; $P>0.10$) or between challenge strains of virus (Mann Whitney; $n_1 = n_2 = 22$, U = 217, $P>0.05$).

In experiment 3 (comparison of ED1 and ED5) swabs were taken only from the nose, and only on 4 days selected to represent virus shedding around the peak levels and during the fall off in the following week. The results are shown in table 5.18. The statistical analysis gives no evidence of any significant difference in virus shedding between calves or between the 2 strains.

Serum Antibody Responses

As noted above, one calf in experiment 1 replicate A was seropositive by both VN and ELISA before the experiment began. Its antibody titre did not rise following the viral inoculation, although it changed from a falling trend to a plateau from 14 days after the inoculation. This calf was omitted from the statistical analysis. All the other calves seroconverted to BHV1 following exposure to the virus. The control calves in experiment 1 remained seronegative to BHV1, indicating that the measures to prevent pen to pen transmission had been successful.

The VN antibody responses of the calves in experiment 1 are shown in fig. 5.7. Although there is some variation in titres between indivi-

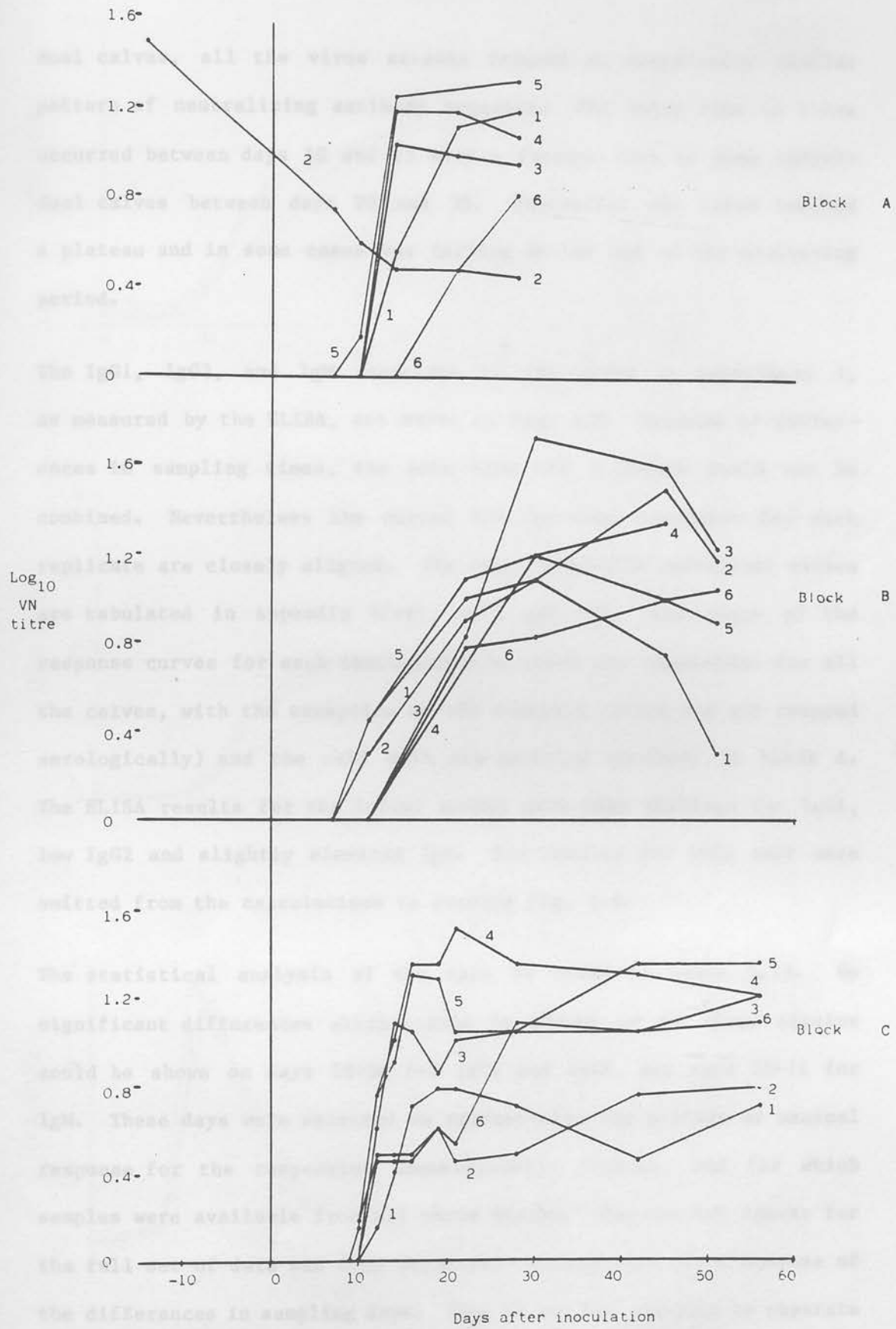


Fig. 5.7 Virus neutralizing antibody responses of individual calves in experiment 1. The labels on each line indicate the virus strain inoculated (ED1 to ED6).

dual calves, all the virus strains induced an essentially similar pattern of neutralizing antibody response. The major rise in titre occurred between days 10 and 15 with a further rise in some individual calves between days 20 and 30. Thereafter the titre reached a plateau and in some cases was falling by the end of the monitoring period.

The IgG1, IgG2, and IgM responses to the virus in experiment 1, as measured by the ELISA, are shown in fig. 5.8. Because of differences in sampling times, the data from the 3 blocks could not be combined. Nevertheless the curves for the mean responses for each replicate are closely aligned. The results for the individual calves are tabulated in appendix 5(vii, viii and ix). The shape of the response curves for each immunoglobulin class was consistent for all the calves, with the exception of the controls (which did not respond serologically) and the calf with pre-existing antibody in block A. The ELISA results for the latter animal gave high readings for IgG1, low IgG2 and slightly elevated IgM. The results for this calf were omitted from the calculations to produce fig. 5.8.

The statistical analysis of the data is shown in table 5.19. No significant differences attributable to blocks or to virus strains could be shown on days 28-30 for IgG1 and IgG2, and days 10-11 for IgM. These days were selected as representing the periods of maximal response for the respective immunoglobulin classes, and for which samples were available from all three blocks. The two-way anovar for the full set of data was done separately within each block because of the differences in sampling days. Thus it was not possible to separate variation due to strain of virus from that due to individual calf

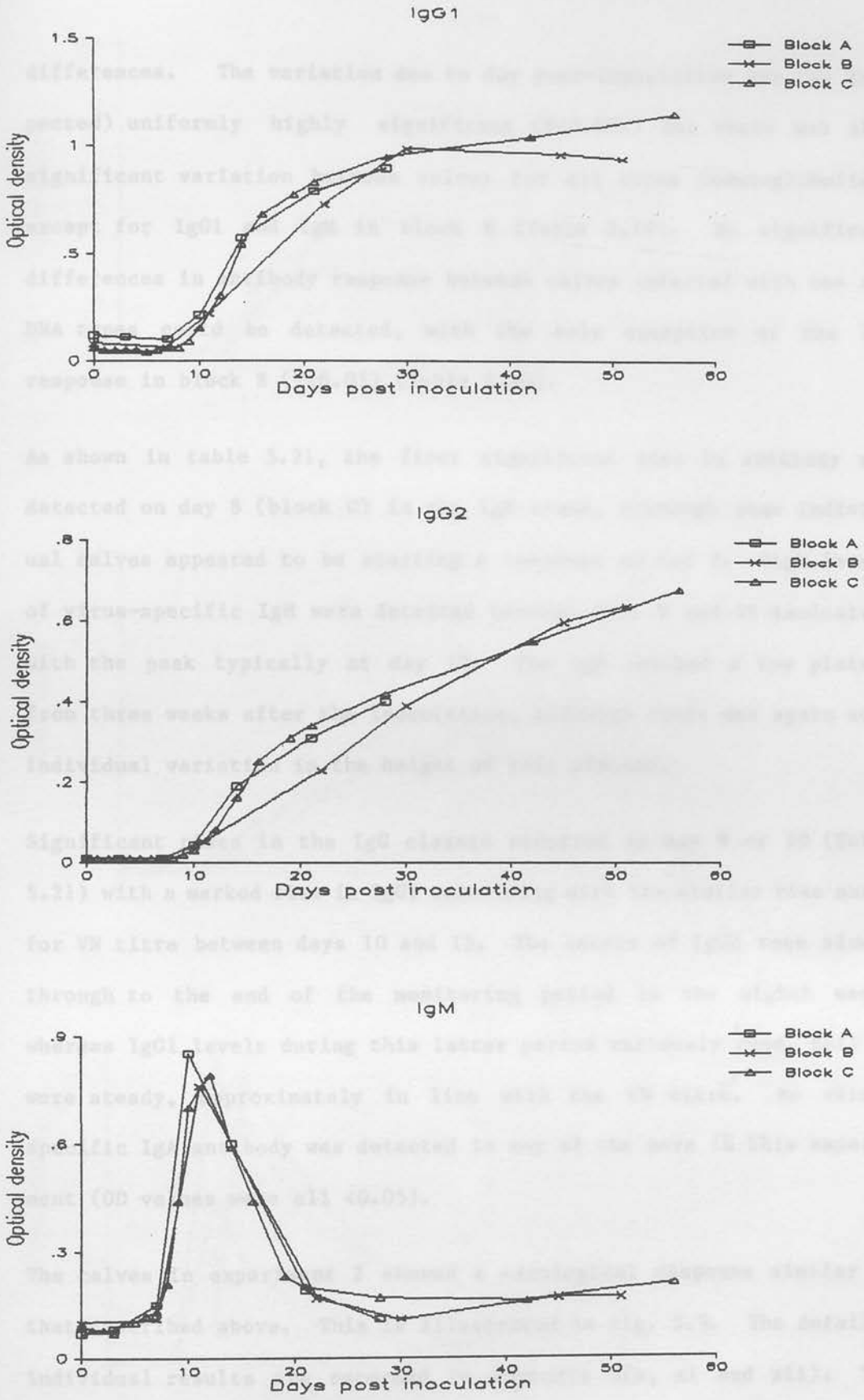


Fig. 5.8 Mean serological responses of calves experimentally infected with BHV1 (experiment 1) as measured in immunoglobulin sub class specific ELISA

differences. The variation due to day post-inoculation was (as expected) uniformly highly significant ($P<0.001$) but there was also significant variation between calves for all three immunoglobulins, except for IgG1 and IgM in block B (Table 5.19). No significant differences in antibody response between calves infected with the two DNA types could be detected, with the sole exception of the IgM response in block B ($P<0.05$) (Table 5.20).

As shown in table 5.21, the first significant rise in antibody was detected on day 8 (block C) in the IgM class, although some individual calves appeared to be starting a response on day 7. High levels of virus-specific IgM were detected between days 9 and 16 inclusive, with the peak typically at day 12. The IgM reached a low plateau from three weeks after the inoculation, although there was again some individual variation in the height of this plateau.

Significant rises in the IgG classes occurred on day 9 or 10 (Table 5.21) with a marked rise in IgG1 coinciding with the similar rise noted for VN titre between days 10 and 15. The levels of IgG2 rose slowly through to the end of the monitoring period in the eighth week; whereas IgG1 levels during this latter period variously rose, fell or were steady, approximately in line with the VN titre. No virus-specific IgA antibody was detected in any of the sera in this experiment (OD values were all <0.05).

The calves in experiment 2 showed a serological response similar to that described above. This is illustrated in fig. 5.9. The detailed individual results are recorded in appendix 5(x, xi and xii). The statistical analysis of the temporal pattern is shown in table 5.22. In the inoculated calves, no significant antibody was detected on day

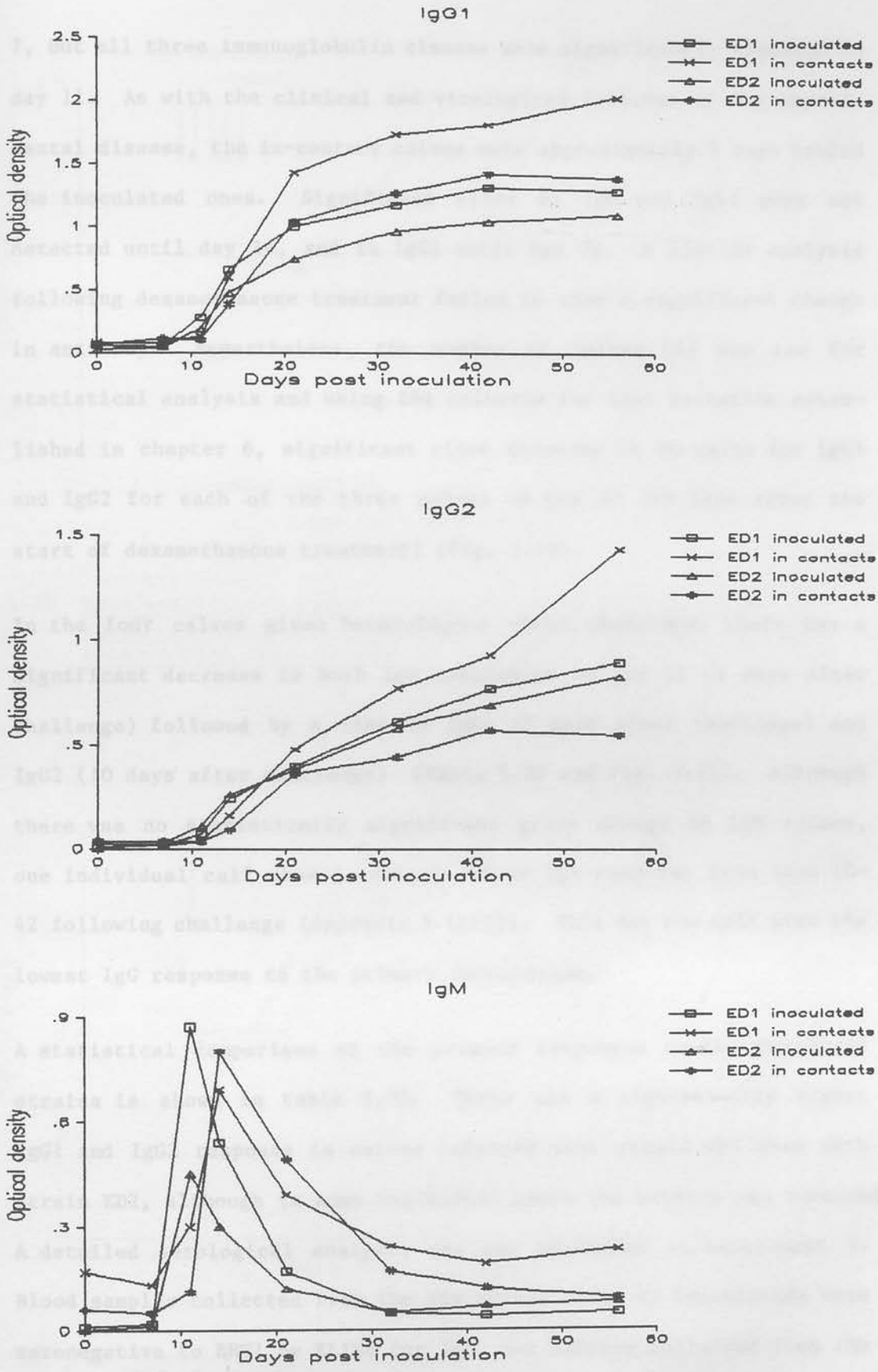


Fig. 5.9 Mean serological responses measured by sub class specific ELISA in calves inoculated or in contact with BHV 1 strains ED1 or ED2 (experiment 2)

7, but all three immunoglobulin classes were significantly elevated on day 11. As with the clinical and virological features of the experimental disease, the in-contact calves were approximately 3 days behind the inoculated ones. Significant rises in IgM and IgG1 were not detected until day 14, and in IgG2 until day 21. A similar analysis following dexamethasone treatment failed to show a significant change in antibody. Nevertheless, the number of calves (3) was low for statistical analysis and using the criteria for test variation established in chapter 6, significant rises occurred in OD value for IgG1 and IgG2 for each of the three calves on day 70 (14 days after the start of dexamethasone treatment) (Fig. 5.10).

In the four calves given heterologous virus challenge, there was a significant decrease in both IgG subclasses on day 92 (4 days after challenge) followed by a rise in IgG1 (7 days after challenge) and IgG2 (10 days after challenge) (Table 5.22 and Fig. 5.11). Although there was no statistically significant group change in IgM values, one individual calf showed evidence of an IgM response from days 10-42 following challenge (Appendix 5 (xii)). This was the calf with the lowest IgG response to the primary inoculation.

A statistical comparison of the primary responses to the two viral strains is shown in table 5.23. There was a significantly higher IgG1 and IgG2 response in calves infected with strain ED1 than with strain ED2, although in some individual pairs the pattern was reversed. A detailed serological analysis was not attempted in experiment 3. Blood samples collected from the six calves prior to inoculation were seronegative to BHV1 by ELISA for IgG, and samples collected from the five surviving calves on day 14 after inoculation were all seropositive, this seroconversion being indicative of a successful inoculation.

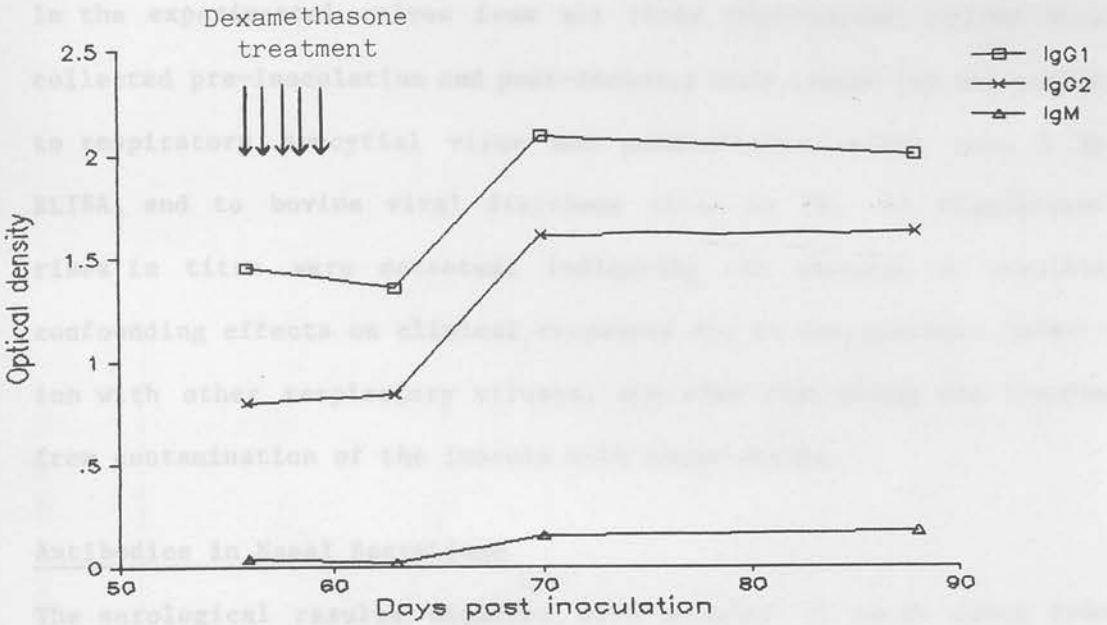


Fig. 5.10 Mean serological responses (by sub class specific ELISA) of three calves recovered from experimental BHV1 infection and treated with dexamethasone on days 56 to 60

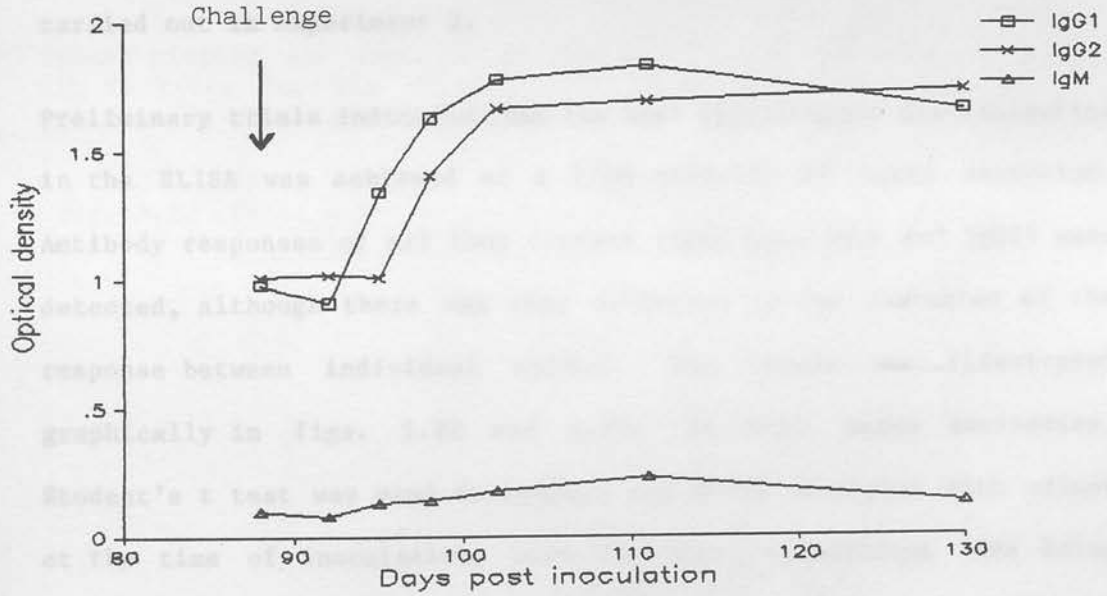


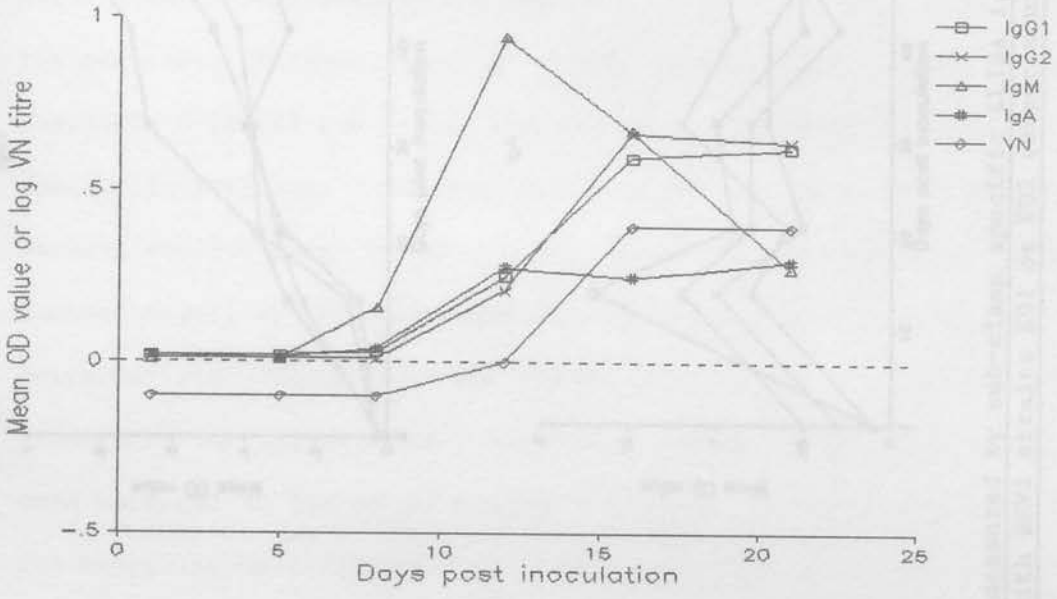
Fig. 5.11 Mean serological responses (by sub class specific ELISA) of four calves following challenge with a heterologous strain of BHV1

In the experimental calves from all three experiments, paired sera collected pre-inoculation and post-recovery were tested for antibodies to respiratory syncytial virus and parainfluenza virus type 3 by ELISA, and to bovine viral diarrhoea virus by VN. No significant rises in titre were detected, indicating the absence of possible confounding effects on clinical responses due to intercurrent infection with other respiratory viruses, and also indicating the freedom from contamination of the inocula with these agents.

Antibodies in Nasal Secretions

The serological results obtained with samples of nasal mucus from experiments 1C and 2 are given in appendix 5 (xiii - xvii). In experiment 1C, virus neutralizing antibody was detected at low titres only, from day 12 onwards. In a number of samples the VN test was unsatisfactory as toxic effects on the cells prevented interpretation at the lower dilutions of mucus. The VN test was not therefore carried out in experiment 2.

Preliminary trials indicated that the best signal-noise discrimination in the ELISA was achieved at a 1/20 dilution of nasal secretion. Antibody responses of all four classes (IgM, IgA, IgG1 and IgG2) were detected, although there was some variation in the character of the response between individual calves. The trends are illustrated graphically in figs. 5.12 and 5.13. As with serum antibodies, Student's t test was used to compare the ELISA responses with values at the time of inoculation, with the first significant rise being detected on day 12 for all four immunoglobulins (Table 5.24). Although not significant by this method, there was evidence of a definite rise in IgM in nasal secretions in at least one calf (inoculated with ED6) on day 8 (Appendix 5 (xiii)), using the criteria for significant rises



Values plotted are mean OD in sub-class specific ELISA or median log VN titre for six calves. For VN an arbitrary value of -0.1 was plotted where all six samples were seronegative.

Fig. 5.12 Local antibody responses in nasal mucus of calves following BHV1 inoculation (experiment 1C)

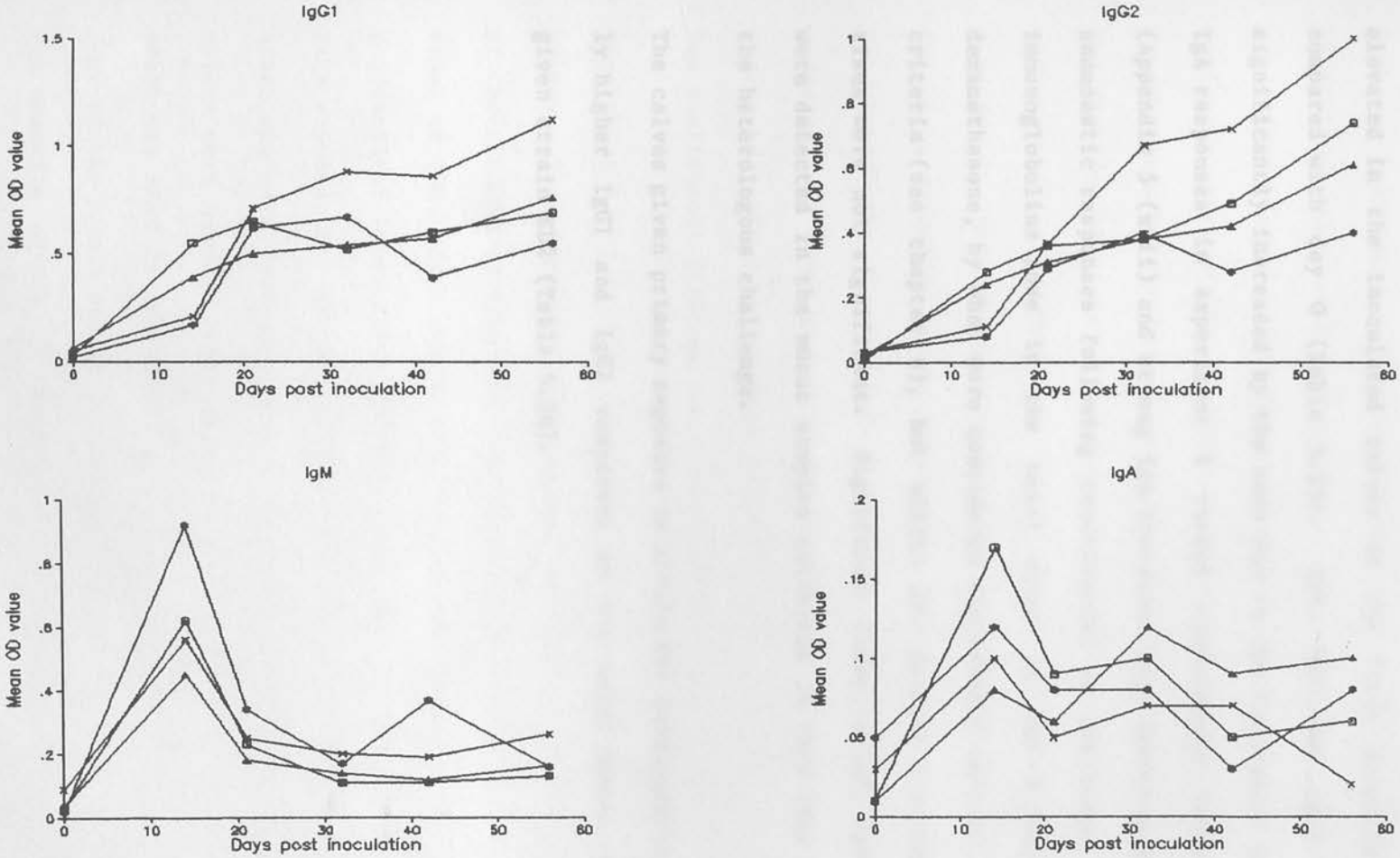


Fig. 5.13 Antibodies in nasal mucus as measured by sub-class specific ELISA in calves inoculated or in-contact with BHV1 strains ED1 or ED2 (experiment 2)

in titre described in chapter 6.

In experiment 2, all four immunoglobulin classes were significantly elevated in the inoculated calves at the first sampling (day 14) compared with day 0 (Table 5.25). IgM, IgG1 and IgG2 were also significantly increased by the same day in the in-contact group. The IgA responses in experiment 2 varied considerably between calves (Appendix 5 (xvii) and strong IgA increases were detected only in the anamnestic responses following reactivation or challenge. All four immunoglobulins rose in the nasal mucus in the 3 calves given dexamethasone, by what were considered significant amounts on general criteria (see chapter 6), but within the present limited data the rises were not significant. Significant rises in IgM, IgG1 and IgG2 were detected in the mucus samples collected 14 days (day 102) after the heterologous challenge.

The calves given primary exposure to strain ED1 developed significantly higher IgG1 and IgG2 responses in the nasal mucus, than those given strain ED2 (Table 5.26).

Experiment 1

Eighteen individual calves were exposed to one of six field isolates of BHV1 by intranasal and unilateral conjunctival inoculation, in a randomized block design. None of the observations showed significant variation attributable to blocks. There was significant variation attributable to the strain of virus, in the clinical responses and the shedding of virus in nasal and left (inoculated) eye swabs. With one exception no significant differences in serum antibody responses were found. When the data were grouped according to the DNA type of the virus inoculum (types 1 and 2 as defined in chapter 4), calves inoculated with type 1 had a significantly more severe clinical response, and shed significantly more virus in the nasal and left ocular secretions, than those given the same dose of type 2 viruses. The statistical findings are summarized in table 5.27.

Experiment 2

Twelve calves were used in an age-matched pair comparison between two of the six BHV1 strains from experiment 1, chosen to represent the two types of DNA. Four pairs were exposed by intranasal and unilateral conjunctival inoculation of the virus and the remaining two pairs were exposed by contact transmission from the inoculated calves. After recovery from the primary infection, the three castrated male calves were treated with dexamethasone to reactivate latent virus, which was shed in both nasal and preputial secretions. Subsequently four of the female calves (ie two pairs) were challenge inoculated with the opposite strain of virus to that they had received on primary exposure.

As in experiment 1, virus of DNA type 1 on primary exposure produced more severe clinical disease, and the calves shed more virus, than with DNA type 2. In addition the IgG1 and IgG2 antibody responses in serum and nasal secretions were higher in the calves exposed to type 1 virus. The statistical tests in support of these findings are summarized in table 5.28. The same trends were observed in both inoculated and contact-infected groups but with an approximately 3 day time lag in the latter case. On heterologous virus challenge, only very mild clinical signs were recorded. There were no significant differences in clinical score or virus shedding attributable to the strain of challenge virus.

Experiment 3

Two groups, each of three Jersey calves, were inoculated with one of two BHV1 strains from experiment 1, both representing type 1 DNA, but in which there was a suspicion of difference in virulence. Neither the clinical scores nor the virus shedding differed significantly between the two strains although the clinical disease was milder than in Friesian calves given the same inoculum in experiment 2, suggesting a difference in breed susceptibility.

Serological responses

Notwithstanding the differences in height of IgG responses noted above in experiment 2, a consistent pattern of humoral and secretory antibody development was observed in all the experiments (Figs. 5.7 - 5.13). A significant rise in antibody was assessed either by a paired t test of ELISA values on sample day compared with day 0 of the treatment, or by the general criteria of test variation (see chapter 6) which allow acceptance as significant of a rise in OD of at least

0.20 between two samples from the same calf.

The earliest serum antibody detected after primary inoculation was IgM on day 8 closely followed by IgG1 and IgG2 on days 9-10. IgM peaked on days 12-14 and was greatly diminished by day 21, while the IgG sub-classes were more persistent. Both IgG1 and virus neutralizing antibody rose sharply between days 10 and 15.

Increases in the IgG subclasses were noted following both dexamethasone-induced recrudescence (day 14 after start of treatment) and secondary exposure to the virus (day 7 after inoculation).

TABLE 5.1 Details of calves used in the pilot study (experiment 1)

Replicate:		A		B		C	
		Sex	Breed	Sex	Breed	Sex	Breed
Virus strain:	ED1	M	FR	F	HX	M	FR
	ED2	M	FR	F	RD	M	FR
	ED3	F	HX	M	FR	M	FR
	ED4	F	HX	F	FR	M	FR
	ED5	M	HX	F	RD	M	FR
	ED6	F	HX	F	HX	M	FR
	Control (1)	M	FR	F	HX	M	FR
	Control (2)	M	FR	-	-	-	-

Key to breeds: FR Friesian type
HX Hereford cross
RD Red-coated dairy type

TABLE 5.2 Details of calves used in experiment 2

Pair No.	Virus Strain: ED1				ED2			
	Calf No	Sex(a)	Age(b)	Treatment(c)	Calf No	Sex(a)	Age(b)	Treatment(c)
1	9366	F	21	C	9370	F	20	C
2	9378	F	16	I	9406	F	15	I
3	9408	F	15	I	9407	F	15	I
4	9409	M	14	I	9412	M	12	I
5	9414	F	12	C	9411	F	13	C
6	9416	F	10	I	9415	M	11	I

Notes: (a) Male calves had been castrated
(b) Age in weeks on the date of inoculation
(c) C= In-contact, I = inoculated

TABLE 5.3 Clinical signs used to evaluate the daily disease scores

Sign		Score range used	Weighting applied
Apathy		0-2	5
Inappetance		0-2	5
Diarrhoea		0-3	2
Dyspnoea		0-2	2
Cough		0-2	2
Adenitis		0-3	2
Rhinitis		0-2	2
Nasal Discharge		0-3	2
Ocular Discharge:	(R eye)	0-3	1
	(L eye)	0-3	1
Conjunctivitis:	(R eye)	0-3	1
	(L eye)	0-3	1
Respiratory Rate (per minute)		<30=0 30-39=1 40-49=2 50-59=3 etc	) 2)))
Rectal Temperature		°C above 39.0°C	10

TABLE 5.4 Cumulative 11 day clinical scores in calves inoculated with six strains of BHV1 (experiment 1)

Virus	DNA Type	Replicate Block A	B	C	Strain mean	Strain median
ED1	1	166	246	166	193	166
ED2	2	(29)[a]	82	78	75[b]	78
ED3	1	207	227	189	208	207
ED4	1	113	127	285	175	127
ED5	1	134	111	51	99	111
ED6	2	85	118	36	80	85
Control		6	5	0	)	
					) 3	2.5
Control		0			)	

Notes (a) Score replaced by an estimated value of 65 for analysis of variance
(b) Using estimated value at (a)

TABLE 5.5 Two way statistical analysis of cumulative clinical scores for experiment 1 (omitting the uninoculated controls)

(i) Anovar

Source of variation	Degrees of Freedom	Sums of Squares	Variance	F	P
Blocks	2	1797	899	0.28	>0.05
Virus strains	5	54389	(10878-963[a]) = 9915	3.11	>0.05
Error	9	28676	3186		
TOTAL	16	84862			

(ii) Friedmann's test

Comparison	k,n	M	P
Blocks	3,6	4.08	>0.10
Virus	6,3	10.81	<0.05

Note (a) Correction because of use of estimated value

TABLE 5.6 Comparison of clinical scores in experiment 1 by viral DNA type

DNA	Type 1	Type 2
Number of calves	12	5
Mean score	169	80
Median score	166	82
Variance	4365	853
Pooled variance	3429	
Student's t (15 d.f.)	2.85 (P<0.02)	
Mann Whitney U	6 (P<0.01)	

NOTE: The seropositive calf (virus ED2, block A) was omitted from this analysis.

TABLE 5.7 Cumulative 15 day clinical scores in age-matched pairs of calves inoculated or in contact with two strains of BHV1 (experiment 2)

Pair No (see table 5.2) :	1		5		2		3		4		6		Mean	
Virus Strain:	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Clinical sign														
Apathy													0	0
Inappetance	5		10		15				5		10		7.5	0
Diarrhoea													0	0
Dyspnoea	4												0.7	0
Cough	2				4	6		6		4	6		2	2.7
Adenitis			2										0.3	0
Rhinitis	24	16	22	10	22	14	30	14	18	22	6	2	20.3	13
Nasal discharge	8	14	32	10	24	20	30	16	28	26	24	10	24.3	16
Ocular discharge:														
R eye	4		21		1		1	3	6	1	4	1	6.2	0.8
L eye			14	11	10	13	16	6	13	5	20	11	12.2	7.7
Conjunctivitis:														
R eye	3		25	6	1	4	4	2	4	4	8	3	7.5	3.2
L eye		4	18	16	6	20	20	13	14	11	25	16	13.8	13.3
Respiratory rate	8	2									2		1.7	0.3
Rectal temperature	44	13	51	32	69	42	73	22	73	58	72	23	63.7	31.7
TOTAL (CS)	102	49	195	85	152	119	174	82	161	131	177	66	160.1	88.6
Difference ($D = CS_1 - CS_2$)	53		110		33		92		30		111		71.5	
Median score													167.5	83.5

Student's t (5 d.f.) for pair differences = 4.68 ($P<0.01$)
Wilcoxon's signed rank test for pair differences, t, T = 0,21 ($P<0.01$)
Mann Whitney test for comparison of virus strain medians, U = 2 ($P<0.05$)

TABLE 5.8 Cumulative 11 day clinical scores in calves challenged with BHV1 of a different genotype from the primary inoculum

Pair No:		2		3	
Original inoculum:		ED1	ED2	ED1	ED2
Challenge virus:		ED2	ED1	ED2	ED1
*Clinical signs:					
Nasal discharge		0	2	0	4
Ocular discharge:	R eye	2	1	0	1
	L eye	1	0	1	0
Rectal temperature		0	5	0	1
TOTAL		3	8	1	6

*Note: Full daily examinations were made, but no clinical signs were observed other than those shown.

TABLE 5.9 Cumulative 11 day clinical scores in calves inoculated with two strains of BHV1 of similar genotypes (experiment 3)

Virus Strain:		ED1			ED5		
Calf No.		54(a)	60	62	53	55	61
Clinical sign (a):							
Rhinitis		2	22	22	12	4	18
Nasal discharge		6	24	24	22	20	12
Ocular discharge:	R eye	0	3	0	0	0	0
	L eye	2	9	10	1	0	6
Conjunctivitis:	R eye	0	1	0	0	0	0
	L eye	1	11	9	0	3	8
Rectal temperature		9	42	35	33	36	60
TOTAL SCORE		20	112	100	68	63	104
Mean score			77			78	

Student's t (4 d.f.) for difference between strain means = 0.032 (P>0.10)

Mann Whitney test for difference between strains (individual scores, 7 observations per calf) $n_1 = n_2 = 21$, U = 194 (P>0.10)

Note (a) calf died, 13 days after inoculation (see text). If the data from this calf is omitted, as an outlier, the differences between strains are still not significant (t = 1.62, P>0.10; $n_1 = 14$, $n_2 = 21$, U = 97, P>0.10)

Table 5.10 Weight of nasal/ocular secretion collected on swabs 8 days after inoculation (experiment 1, replicate B)

Animal	Nasal mucus (g)	Right lachrymal secretion (g)	Left lachrymal secretion (g)
1	0.15	0.04	0.07
2	0.17	0.05	0.04
3	0.22	0.03	0.07
4	0.14	0.01	0.06
5	0.11	0.02	0.03
6	0.09	0	0.01
7	0.11	0.04	0.05
Mean	0.14	0.03	0.05
Standard deviation	0.04	0.02	0.02
Mean dilution (w/v) in 3 ml extraction medium	1/21	1/100	1/60

TABLE 5.11 Cumulative virus titres ($log_{10}TCID_{50}$) for days 1-11 inclusive after inoculation (experiment 1)

Virus	DNA Type	Block A	Block B	Block C
ED1	1	28.50	30.25	21.25
ED2	2	22.25	17.50	17.50
ED3	1	32.00	27.00	25.00
ED4	1	28.50	24.25	25.50
ED5	1	30.25	26.50	20.50
ED6	2	12.00	16.50	18.75
Control		0	0	0
Control		0		

Two way analysis by Friedmann's test (excluding the control calves)

Comparison	k, n	M	P
Blocks	3,6	2.58	>0.10
Strains	6,3	11.24	<0.05

Comparison of DNA types by Mann Whitney test

DNA type	No. observations	Median cusum titre	U	P
1	12	26.75	2	<0.001
2	6	17.50		

TABLE 5.12 Analysis of variation for daily virus titres in nasal swabs ($log_{10}TCID_{50}$ per 90µl extraction medium) using Kruskal-Wallis test (experiment 1)

Source of variation		Days 1 - 11				Days 1 - 7			
		Median titre	n	H	P	Median titre	n	H	P
Block:	A	1.875	66)			3.875	42)		
	B	1.750	66)	1.18	>0.10	3.375	42)	3.30	>0.10
	C	2.125	66)			3.250	42)		
Virus:	ED1	2.50	33)			4.00	21)		
	ED2	1.50	33)			2.50	21)		
	ED3	2.50	33)	8.83	>0.10	3.75	21)	15.14	<0.01
	ED4	2.50	33)			3.25	21)		
	ED5	1.75	33)			4.00	21)		
	ED6	0.75	33)			2.50	21)		
DNA type:	1	2.500	132)	8.31	<0.01	3.75	84)	13.56	<0.001
	2	0.875	66)			2.50	42)		
Day:	1	2.00	18)			2.00	18)		
	2	4.87	18)			4.87	18)		
	3	3.75	18)			3.75	18)		
	4	3.75	18)			3.75	18)	66.54	<0.001
	5	4.00	18)			4.00	18)		
	6	2.75	18)	154.3	<0.001	2.75	18)		
	7	1.37	18)			1.37	18)		
	8	0	18)						
	9	0	18)						
	10	0	18)						
	11	0	18)						

n = number of observations

H = Kruskal-Wallis statistic (adjusted for ties)

TABLE 5.13 Virus isolation from ocular swabs in experiment 1. Median daily titres ($log_{10}TCID_{50}$ per 90µl extraction medium) and cumulative titres for days 1-11 after inoculation

		L eye				R eye		
		Cusum titres				Cusum titres		
		Block			Daily	Block		Daily
Virus	DNA type	A	B	C	median titre	B	C	median titre
ED1	1	21.00	16.75	17.75	1.25	9.25	0	0
ED2	2	8.75	8.00	10.00	0	1.75	1.50	0
ED3	1	27.25	11.50	19.00	1.50	2.00	7.75	0
ED4	1	21.00	13.75	24.50	1.75	4.25	4.25	0
ED5	1	23.00	14.50	15.75	1.00	0	0.50	0
ED6	2	4.50	13.25	6.75	0	3.00	0.50	0

Two way analysis of Cusum titres by Friedmann's test

Comparison	k, n	M	P
L eye blocks	3, 6	4.00	>0.10
L eye strains	6, 3	8.67	>0.10
R eye blocks	2, 6	too few to analyse	
R eye strains	6, 2	3.79	>0.10

Analysis of daily titres by Kruskal Wallis test

Comparison	k, n	H	P
L eye blocks	3, 66	2.12	>0.10
L eye strains	6, 33	14.61	<0.05
R eye blocks	2, 66	0.04	>0.10
R eye strains	6, 22	9.66	>0.05

Comparison of cusum titres by DNA type using Mann Whitney test

Eye	DNA	n	Median cusum titre	U	P
L	1	12	18.375)	1	<0.001
L	2	6	8.375)		
R	1	8	3.125)	12.5	>0.10
R	2	4	1.625)		

Notes
Right eyes were not swabbed in block A
k = number of levels in the analysis
n = number of observations per level

TABLE 5.14 Patterns of virus shedding in nasal secretions (experiment 2)

Notes: - Tabulated values are log10 $TCID_{50}$ per 90µl extraction medium.

- A value of <1 indicates no growth of virus in the 1/10 dilution, where it was not possible to evaluate the wells with undiluted sample due to toxic effects on the cells. An arbitrary value of 0 was assigned.

- A reading of '-' indicates no growth of virus in the wells with undiluted sample, also assigned the value 0.

	In-contacts				Inoculated							
Pair No	1		5		2		3		4		6	
Virus strain	1	2	1	2	1	2	1	2	1	2	1	2
Days after inoculation: 1	<1	<1	<1	<1	<1	<1	1.25	<1	1.25	1.0	<1	0.75
2	<1	<2	<2	<1	4.5	3.5	1.75	<1	2.5	2.75	2.5	1.0
3	<1	<1	<1	<1	4.75	<1	3.75	1.75	4.0	3.0	1.5	<1
4	0.6	<1	<1	2.0	4.0	3.5	4.5	3.5	4.75	3.0	3.5	1.75
5	1.5	1.75	4.5	2.25	3.25	3.5	4.0	3.5	4.0	3.5	3.75	3.5
6	4.5	2.75	4.25	3.75	3.5	2.0	3.25	2.25	4.0	2.25	3.25	3.75
7	4.75	3.25	5.5	3.75	3.5	2.0	3.25	1.75	3.25	1.75	2.5	2.5
8	3.75	3.5	4.5	2.5	<1	0.75	2.5	1.0	2.0	1.75	1.0	1.0
9	3.5	3.5	5.5	3.5	0.75	0.6	<1	0.6	1.5	1.25	<1	0.75
10	3.0	3.25	4.75	2.0	0.3	<1	-	<1	0.75	0.45	<1	0.6
11	0.75	1.5	2.0	0.15	<1	<1	<1	<1	<1	0.6	<1	<1
12	<1	1.25	1.5	<1	<1	<1	<1	<1	<1	<1	<1	<1
13	-	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
14	-	-	<1	-	-	-	<1	-	-	-	<1	<1
15	-	<1	-	-	-	-	-	-	-	-	-	-
Cumulative titre	22.35	20.75	32.50	19.90	24.55	15.85	24.25	14.35	28.00	21.30	18.00	15.60
Difference in cumulative titres (strain 1 - strain 2)	1.60		12.60		8.70		9.90		6.70		2.40	
Wilcoxon's signed rank test for daily titres in each pair												
No. of Differences	8		9		9		9		11		8	
t, T	15,21		6,39		6,39		2,43		10,56		14,22	
P	>0.10		>0.05		>0.05		<0.05		<0.05		>0.10	

Median cusum titre, strain ED1 = 24.40
strain ED2 = 17.88
Mann Whitney test to compare strain medians, U = 3 (P<0.05)
Wilcoxon's signed rank test on cusum pair differences, t, T = 0,21 (P<0.05)
Mann Whitney test to compare virus strains by daily titres, $n_1 = n_2 = 90$,
U = 3627 (P>0.10)

TABLE 5.15 Cumulative virus titres for ocular swabs in experiment 2

Method of infection	Pair	Virus	L eye	R eye
Contact	1	1	0	5.60
		2	1.20	0.90
	5	1	12.25	15.05
		2	11.00	2.35
Inoculation (nose + L eye)	2	1	6.50	1.60
		2	8.95	0.90
	3	1	12.50	2.25
		2	9.80	1.50
	4	1	9.75	6.00
		2	3.00	3.50
	6	1	10.25	6.10
		2	7.50	2.55

Inoculated eyes (L eye, pairs 2, 3, 4, 6)

Wilcoxon signed rank test (for differences ED1 - ED2):
t, T = 1, 9 ($P>0.10$)

Mann Whitney comparison of medians ED1 vs. ED2:
$n_1 = n_2 = 4$, $U = 4$ ($P>0.05$)

Non-inoculated eyes (all R eyes, plus L eye, pairs 1 and 5)

Wilcoxon signed rank test (for differences ED1 - ED2):
t, T = 3, 33 ($P<0.05$)

Mann Whitney comparison of medians ED1 vs ED2:
$n_1 = n_2 = 8$, $U = 19$ ($P>0.10$)

TABLE 5.16 Recovery of BHV1 from calves following reactivation with dexamethasone (experiment 2)

Calf		9409		9412		9415	
Original virus inoculum		ED1		ED2		ED2	
Swab site		Nose	Prepuce	Nose	Prepuce	Nose	Prepuce
Day	0*	-	-	-	-	-	-
	1*	+	+	+	+	-	+
	2*	+	+	+	+	+	+
	3*	+	+	+	+	-	+
	4*	+	+	+	+	+	+
	5	+	+	+	+	-	-
	6	+	+	+	+	+	+
	7	+	-	+	+	-	+
	8	+	-	+	+	+	+

*dexamethasone injected on these days

TABLE 5.17 Virus shedding ($TCID_{50}$ per 90µl extraction medium) in calves following heterologous BHV1 challenge (experiment 2)

Pair		2						3					
Calf		9378			9406			9408			9407		
Original inoculum		ED1			ED2			ED1			ED2		
Challenge inoculum		ED2			ED1			ED2			ED1		
Days after challenge		N	R	L	N	R	L	N	R	L	N	R	L
	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	3.25	-	-	2.5	-	-	1.5	-	-	0.75	-	-
	2	2.0	-	0.15	4.5	-	-	3.5	-	-	3.0	-	-
	3	3.75	-	2.25	4.0	-	-	1.75	-	-	3.25	-	-
	4	1.75	-	0.45	4.5	-	0.15	3.0	-	-	3.5	-	-
	5	3.0	-	-	3.75	-	-	3.0	-	-	2.75	-	-
	6	1.5	-	-	2.5	-	-	-	-	-	2.25	-	-
	7	0.15	-	-	0.75	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	0.6	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-	-	-
	10	0.6	-	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-	-	-	-
cumulative titre		16.00	0	2.85	22.50	0	0.15	13.35	0	0	15.50	0	0

N = nasal swab
R, L = right and left eye swabs

TABLE 5.18 Comparison of nasal shedding of virus in calves inoculated with 2 strains of BHV1 of similar genotype (experiment 3)

Titres are expressed in log $TCID_{50}$ per 90µl extraction medium.

Virus:	ED1			ED5		
Calf :	x54	x60	x62	x53	x55	x61
Days after inoculation 4	2.25	3.0	3.25	4.0	2.25	2.25
7	4.5	4.0	3.75	2.5	<1	3.5
9	<1	2.25	1.75	<1	<1	0.75
11	<1	<1	<1	<1	<1	<1
Cumulative titre	6.75	9.25	8.75	6.5	2.25	6.5
Median cumulative titre		8.75			6.5	

Friedmann's test for differences in daily titres between calves:
k, n = 6,4; M = 4.68 (P>0.10)

Mann Whitney comparison of daily titres between virus strains:
$n_1 = n_2 = 12$, U = 76 (P>0.10)

TABLE 5.19 Analysis of variance of ELISA values for serum antibody in experiment 1.

Data set	Source of variation	d.f.	IgG1 SS	IgG1 MS	IgG1 F	IgG1 P	IgG2 SS	IgG2 MS	IgG2 F	IgG2 P	IgM SS	IgM MS	IgM F	IgM P
Day of peak titre[a]	Block	2	0.066	0.033	0.97	>0.05	0.002	0.001	0.04	>0.05	0.047	0.023	0.38	>0.05
	Virus[b]	5	0.447	0.072	2.12	>0.05	0.151	0.030	1.20	>0.05	0.936	0.182	2.98	>0.05
	Error	9	0.309	0.034			0.223	0.025			0.548	0.061		
	Total	16	0.822				0.376				1.531			
Block A	Calf + virus [c]	4	0.180	0.045	6.18	<0.01	0.051	0.013	3.71	<0.05	0.349	0.087	3.52	<0.05
	Day	6	3.566	0.594	81.52	<0.001	0.797	0.133	38.62	<0.001	2.790	0.465	18.75	<0.001
	Error	24	0.175	0.007			0.083	0.003			0.596	0.025		
	Total	34	3.921				0.931				3.735			
Block B	Calf + virus	5	0.404	0.081	2.25	>0.05	0.418	0.084	5.96	<0.001	0.044	0.009	0.56	>0.05
	Day	6	6.817	1.136	31.65	<0.001	2.694	0.449	30.54	<0.001	2.015	0.336	21.39	<0.001
	Error	30	1.076	0.036			0.441	0.015			0.472	0.016		
	Total	41	8.297				3.553				2.531			
Block C	Calf + virus	5	0.988	0.198	12.51	<0.001	0.484	0.097	7.38	<0.001	1.127	0.225	9.43	<0.001
	Day (2,4,6 omitted)	12	12.479	1.040	65.82	<0.001	3.739	0.312	23.79	<0.001	3.855	0.321	13.44	<0.001
	Error	60	0.949	0.016			0.786	0.013			1.437	0.024		
	Total	77	14.416				5.008				6.418			

Notes (a) Approximately - ie days 28 or 30 (IgG) and 10 or 11 (IgM).
(b) Estimated values for ED2 in block A: IgG1, 0.57; IgG2, 0.42; IgM, 0.67
(c) Omitting calf infected with ED2

TABLE 5.20 Comparison of antibody responses measured by ELISA according to DNA type of the virus (experiment 1)

Block	Days tested	No. observations		Student's t statistic (and significance)		
		DNA 1	DNA 2	IgG1	IgG2	IgM
A	3 - 28	24	6	1.40 (NS)	0.94 (NS)	0.85 (NS)
B	7 - 51	24	12	0.33 (NS)	0.13 (NS)	2.10 (<0.05)
C	7 - 56	48	24	1.96 (NS)	1.38 (NS)	0.91 (NS)

Notes: A pooled variance estimate for the two DNA types was used for each analysis.

NS = not significant (P>0.05)

TABLE 5.21 Values of Student's t statistic and probability levels (P) comparing mean ELISA values for serum antibodies on sampling days post-inoculation with those on the day of inoculation (experiment 1).

		IgG1		IgG2		IgM	
Block	Day	t	P	t	P	t	P
A	3	-0.38	NS	-1.00	NS	0.59	NS
	7	-0.71	NS	-1.00	NS	1.68	NS
	10	3.54	<0.05	5.49	<0.01	4.23	<0.05
	14	6.08	<0.01	6.29	<0.01	3.89	<0.05
B	7	-1.46	NS	1.00	NS	1.10	NS
	11	4.57	<0.01	4.47	<0.01	5.74	<0.01
	22	8.02	<0.001	4.66	<0.01	1.48	NS
	30	7.77	<0.001	5.49	<0.01	0.72	NS
C	7	0	NS	1.00	NS	2.28	NS
	8	0.55	NS	1.46	NS	3.43	<0.05
	9	1.70	NS	2.71	<0.05	4.10	<0.01
	10	4.17	<0.01	4.18	<0.01	4.22	<0.01
	12	5.29	<0.01	6.01	<0.01	3.61	<0.05

NS = not significant (P>0.05)

TABLE 5.22 Values of Student's t statistic and probability levels (P) for the mean difference in ELISA values for serum antibodies between day of sample (D_S) and day of treatment (D_T) in experiment 2

No. Calves	Treatment	Days		IgG1		IgG2		IgM	
		D_T	Ds	t	p	t	p	t	p
4	Contact exposure	0	7	1.36	NS	-1.00	NS	-0.93	NS
		0	11	1.51	NS	0.52	NS	1.59	NS
		0	14	4.43	<0.05	2.94	NS	7.79	<0.01
		0	21	6.18	<0.01	6.99	<0.01	3.73	<0.05
8	Inoculated	0	7	-0.15	NS	1.56	NS	1.51	NS
		0	11	6.56	<0.001	4.02	<0.01	5.28	<0.001
		0	14	7.94	<0.001	9.27	<0.001	5.31	<0.001
		0	21	8.06	<0.001	9.22	<0.001	8.44	<0.001
3	Dexamethasone	56	63	-1.96	NS	0.70	NS	-1.00	NS
		56	70	2.45	NS	2.98	NS	3.21	NS
		56	88	1.70	NS	3.36	NS	2.32	NS
4	Heterologous challenge	88	92	-3.74	<0.05	-3.34	<0.05R	-0.64	NS
		88	95	5.06	<0.05	-0.15	NS	1.28	NS
		88	98	7.43	<0.01	8.74	<0.01	0.77	NS
		88	102	8.88	<0.01	7.84	<0.01	1.28	NS

NS = not significant (P>0.05)
R = significant decrease in OD value

TABLE 5.23 Paired comparison of serum antibody responses to primary challenge with virus strains ED1 or ED2 (experiment 2)

	IgG1	IgG2	IgM
Sample days	11-56	11-56	11-21
No. pair differences	36	36	18
No. pair differences ≠ 0 (for Wilcoxon test)	35	34	17
Wilcoxon t, T	91.5, 538.5	178.5, 416.5	52, 101
P	<0.001	<0.05	>0.10
Student's t	4.64	2.40	1.99
P	<0.001	<0.05	>0.05

TABLE 5.24 Values of Student's t statistic and probability levels (P) for the mean difference in ELISA values for nasal secretory antibodies between day of sample and day 1 after inoculation in experiment 1C.

	IgG1		IgG2		IgM		IgA	
Day	t	P	t	P	t	P	t	P
5	0	>0.10	-1.00	>0.10	-0.85	>0.10	-2.39	>0.05
8	2.24	>0.05	-1.11	>0.10	1.99	>0.05	1.28	>0.10
12	4.17	<0.01	5.75	<0.01	3.69	<0.05	5.04	<0.01

TABLE 5.25 Values of Student's t statistic and probability levels (P) for the mean difference in ELISA values for nasal secretory antibodies between day of sample (D_S) and day of treatment (D_T) in experiment 2.

		Days		IgG1		IgG2		IgM		IgA	
No. Calves	Treatment	D_T	D_S	t	P	t	P	t	P	t	P
4	Contact exposure	0	14	17.86	<0.001	6.48	<0.01	4.33	<0.05	2.40	>0.05
		0	21	7.22	<0.01	12.29	<0.01	4.54	<0.05	1.51	>0.10
		0	32	13.33	<0.001	5.93	<0.01	7.54	<0.01	2.33	>0.05
		0	42	4.26	<0.05	3.54	<0.05	2.76	>0.10	0.30	>0.10
		0	56	4.55	<0.05	3.74	<0.05	5.14	<0.05	1.00	>0.10
8	Inoculated	0	14	6.23	<0.001	10.18	<0.001	9.50	<0.001	4.34	<0.01
		0	21	6.31	<0.001	14.28	<0.001	6.52	<0.001	6.48	<0.001
		0	32	6.30	<0.001	12.09	<0.001	4.76	<0.001	3.67	<0.01
		0	42	5.14	<0.001	8.99	<0.001	4.32	<0.01	3.01	<0.05
		0	56	12.44	<0.001	10.83	<0.001	4.99	<0.001	2.77	<0.05
3	Dexamethasone	56	63	-0.86	>0.10	-0.30	>0.10	-0.48	>0.10	-0.64	>0.10
		56	70	1.82	>0.10	2.27	>0.10	2.30	>0.10	1.75	>0.10
		56	88	1.18	>0.10	1.26	>0.10	0.50	>0.10	1.31	>0.10
4	Heterologous challenge	88	102	7.86	<0.01	5.58	<0.05	10.08	<0.01	2.25	>0.10

TABLE 5.26 Paired comparison of nasal secretory antibody responses to primary challenge with virus strains ED1 or ED2 (experiment 2)

Sample days: 14-56
No. pair differences (ED1-ED2): 30

	Student's t (29 d.f.)	P
IgG1	2.80	<0.01
IgG2	3.84	<0.001
IgM	0.43	>0.10
IgA	0.19	>0.10

TABLE 5.27 Summary of statistical comparisons in experiment 1

	Type of Analysis	Source of Variation		
		Blocks	Virus strains	Virus DNA type
Clinical score	a	NS	NS	P<0.05
	b	NS	P<0.05	P<0.001
Virus titre excreted				
- nasal cusum	b	NS	P<0.05	P<0.001
- nasal daily (days 1-7)	b	NS	NS	P<0.001
- L eye cusum	b	NS	NS	P<0.001
- R eye cusum	b	X	NS	NS
- L eye daily	b	NS	P<0.05	X
- R eye daily	b	NS	NS	X
Serum antibody response				
- IgG1	a	NS	NS	NS
- IgG2	a	NS	NS	NS
- IgM	a	NS	NS	S

Notes: a parametric tests
b non-parametric tests
NS not significant (P>0.05)
S significant (P<0.05) for one block only
X not analysed

TABLE 5.28 Summary of statistical comparisons between virus strains in experiment 2

Variable	Type of Analysis	Primary Inoculation	Challenge Inoculation
Clinical score	a	P<0.01	X
	b	P<0.05	NS
Virus titre in swabs:			
nasal cusum	b	P<0.05	X
nasal daily	b	NS	NS
inoculated eyes	b	NS	X
uninoculated eyes	b	P<0.05	X
Serum antibody:			
IgG1	a	P<0.001	X
IgG2	a	P<0.05	X
IgM	a	NS	X
Nasal secretory antibody:			
IgG1	a	P<0.01	X
IgG2	a	P<0.001	X
IgM	a	NS	X
IgA	a	NS	X

NOTES: a parametric tests
b non-parametric tests
NS not significant (P>0.05)
X not analysed

Chapter 6

ASSESSMENT OF DIAGNOSTIC METHODS FOR INFECTIOUS BOVINE RHINOTRACHEITIS

INTRODUCTION

The specific diagnosis of IBR may be said to have begun with the first recorded *in vitro* isolation of the virus from diseased animals in the USA (Madin and others, 1956). Virus isolation in cell culture remains a useful diagnostic method, although in recent years its importance has diminished as developments in immunoassay technology have liberated viral diagnosis from dependence on the constant availability of cell cultures. Samples received at the Central Veterinary Laboratory from natural cases of suspected IBR, as well as materials from experimentally infected calves, were used to compare and contrast a variety of diagnostic techniques for the disease. The two major areas investigated were the detection of viral antigens and the detection of the hosts' responses by serology. Because the chapter is largely concerned with optimisation of the methodology, the materials, methods and results have been combined under each sub-heading.

EXPERIMENTAL ANIMALS

Some materials were collected from the animals in experiments 1 and 2 (described in chapter 5). In addition, two further series of animal experiments were conducted. In experiment 4, three 6-7 month old Friesian calves, seronegative to BHV1, were inoculated intranasally with a single dose of 4×10^8 $TCID_{50}$ of virus strain ED4. Three control calves in an adjacent but separate pen were inoculated with

non-infected cell culture harvest. All six animals were examined daily although full clinical scoring was not carried out. The three infected calves developed a febrile upper respiratory disease, (similar to that described in chapter 5) with elevated rectal temperature on days 2 to 6 or 7 inclusive. Short cotton wool nasal swabs were taken daily, as described in chapter 5. In addition, on days 1 to 3 after inoculation, long nasopharyngeal brush swabs, as described by Thomas and Stott (1975), were taken. Both swab types were eluted into 3 ml PBSL for transport to the laboratory.

Experiment 5 was an investigation into possible interactions between bovine virus diarrhoea virus and BHV1 vaccination. It has been fully described elsewhere (Edwards and others, 1986a). The final part of the experiment consisted of challenging the 24 male Jersey calves with virulent BHV1 (strain ED1) using the same dose of virus and routes of inoculation as in experiments 2 and 3. Ten of the calves had previously been vaccinated intranasally with live temperature-sensitive vaccine against BHV1 (Tracherine, Smith Kline Animal Health).

One of the non-vaccinated animals (No. 62) appeared to have acquired vaccine virus infection by lateral spread, as evidenced by its antibody response, titre of virus shed and clinical score. With this exception, the non-vaccinates developed clinical signs of IBR following challenge, with a pattern of disease similar to that reported for the other experiments. As also noted for experiment 3 (chapter 5), the severity of disease, indicated by the clinical scores (Edwards and others 1986a), was less in these Jersey calves than in the Friesians used for the earlier experiments. In the comparison, for virus ED1 only, between clinical scores in experiment 2 (Friesians) and

experiment 5 (Jerseys), the Mann Whitney U statistic was 1 (n_1 = 13, n_2 = 6, $P<0.001$); but comparing experiments 3 and 5 (both Jerseys), U = 14 (n_1 = 13, n_2 = 3, $P>0.10$). Clinical signs in the vaccinated calves for experiment 5 were transient or absent. Short swabs were collected as described above, during the post-challenge period, and used for further assessment of antigen detection methods.

COMPARISON OF SAMPLE PREPARATION METHODS FOR ANTIGEN DETECTION

In experiment 1, blocks A and B, smears of nasal secretion were prepared by wiping freshly-collected swabs across glass microscope slides and allowing the mucus smears to air-dry. These were subsequently fixed in cold acetone then stained by the direct fluorescent antibody method (see below). On examination by fluorescence microscopy, a confident diagnosis of BHV1-specific fluorescence was made on samples collected on days 2, 3 and 4 after inoculation, but beyond this period considerable doubt entered into the interpretation due to a paucity of positively stained cells and high, confusing background fluorescence. An attempt to enhance the specific fluorescence on duplicate smears by using the indirect staining method (unlabelled bovine antiserum followed, after thorough washing, by rabbit anti-bovine IgG FITC conjugate (Nordic)) also increased the background fluorescence and worsened the perceived signal/noise ratio.

Samples from experiment 4 (which chronologically preceded experiments 1C, 2 and 3) were used to establish a better method of cytological smear preparation, which was used in the remaining animal experiments and subsequently recommended for field diagnostic use. Nasal swabs, immediately after collection, were broken off into (or agitated vigorously in) 3 ml aliquots of PBSL which were transported to the

laboratory in small screw-capped bottles. After agitation on a vortex mixer the swabs if still present were removed and the samples centrifuged at 500g for five minutes. The supernatant was reserved for virus isolation, or detection of soluble antigens. The preparation of cell smears was based on the methods of Minnich and Ray (1980) and Thomas and Stott (1981). The cell deposit was mixed with 1 ml PBS (for short nasal swabs) or 5 ml PBS (for long, brush swabs) and single drops were spotted onto PTFE coated multispot glass slides (C.A. Hendley Ltd) and evaporated to dryness. The slides were fixed in cold (4°C or -20°C) acetone for 10 minutes and rinsed in distilled water before immunolabelling.

The cell suspension could be further washed in PBS before spotting onto the slide but this was found to confer little advantage. An alternative procedure assessed was the centrifugation of cells from the sample suspension directly onto glass slides using a 'cytospin 2' centrifuge (Shandon) operating at 400g for 5 minutes. The preparations were as good as, but no better than, the simple drying technique. The cytospin method was also more cumbersome, requiring cleaning of the apparatus after each centrifugation; and produced only one spot smear per slide, compared with 12 by the drying technique.

The washed-cell, dried-smear technique gave very satisfactory preparations, with a good density of cells for examination (Figs 6.1-6.3) and low background labelling, enabling the detection of small numbers of infected cells such as were observed as the acute phase of infection regressed, typically on days 6 and 7 after inoculation. In the experimental intranasally-inoculated calves, the long brush swabs generally gave poorer preparations and fewer positive cells than the short

cotton wool swabs. Subsequent field reports from veterinary investigation centres (personal communications) have indicated satisfactory results with long wire and cotton wool swabs ('Laryngeal Swabs', Medical Wire Co.) which are finer and less damaging to the epithelial tissues than the brush swabs.

CYTOLOGICAL LABELLING METHODS FOR ANTIGEN DETECTION

Gnotobiotic bovine immunoglobulins specific for BHV1, conjugated to either FITC or peroxidase and stored at -20°C as 50% glycerolised stocks, were further diluted 1 in 4 (immunofluorescence) or 1 in 5 (immunoperoxidase) respectively, in PBS, initially at pH 7.2, but latterly at pH 7.6 which was found to give more consistent results with immunofluorescence. The optimum dilution of each batch of conjugate was determined by close interval titrations (e.g. 1/3, 1/4, 1/5, 1/6, 1/8, 1/10, 1/12) to establish the highest dilution which gave strong labelling on known positive material (virus-infected cell cultures) together with acceptably low background signal on known negative samples.

The diluted conjugates were reacted with the fixed cell smears for 30 minutes in a humid chamber at either 37°C or room temperature; both gave satisfactory results. Washing was carried out for 10 minutes in each of three changes of PBS. Fluorescent-labelled smears were then counterstained with Evans blue 1/100,000 for one minute, rinsed then mounted in buffered 50% glycerol (pH 7.6) for examination under incident light fluorescence (50W mercury vapour light source, x 40 objective lens). Immunoperoxidase-labelled smears were reacted with DAB substrate mixture for 10 minutes, rinsed, lightly counterstained with toluidine blue, dehydrated and mounted in DPX medium for

examination by bright field microscopy. Specificity of the immunoperoxidase label was monitored by staining duplicate smears with conjugates specific for other viruses.

Both fluorescent and enzyme labels enabled a confident diagnosis of BHV1 infection to be made on samples collected on days 2 to 5 inclusive after inoculation of the experimental animals. Control uninfected animals were uniformly negative. The microscopic picture is illustrated in figs. 6.1 to 6.3. Beyond this period a diagnosis became progressively more difficult to establish, as the number of positively labelled cells in the smears diminished, and then the intensity of labelling became reduced. The results for experiment 1C and experiment 2 are shown in tables 6.1 and 6.2. The positive results were accumulated for each calf, using a scoring system as shown in the tables, to produce a cusum value representing the number of days on which a positive result was found, weighted for the degree of positivity recorded for each sample. The scores were analysed by a paired t-test, comparing immunofluorescence with immunoperoxidase (no significant difference, Table 6.1) and virus strain ED1 with ED2 (significantly higher scores for ED1, $P<0.05$, Table 6.2). In experiment 4 an estimate of the proportion of cells in the smears, labelled positively for viral antigen, was made by differential cell counting of the immunoperoxidase preparations, as shown in table 6.3. The peak values for positively labelled cells occurred on day 3.

In the immunoperoxidase system no problem was encountered, in the experimental animals, with endogenous cellular enzymes yielding "false positive" coloured reaction products. If present this would have appeared equally in test and control smears. In view of the accepted risk of this phenomenon (Benjamin, 1979) the effect of enzyme block-

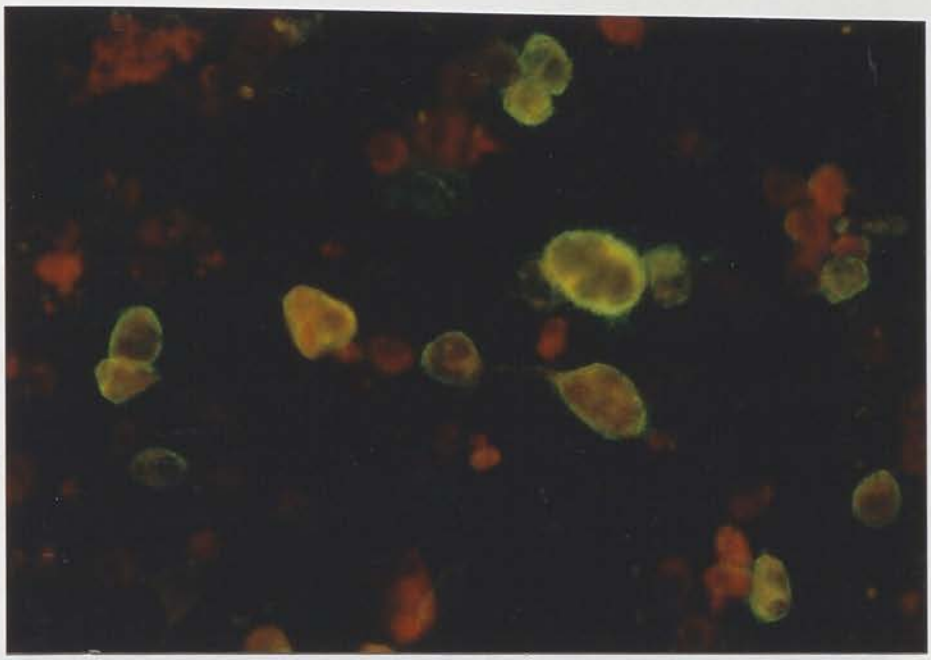


Fig. 6.1 BHV1 antigen in nasal epithelial cells of an experimentally infected calf, labelled by direct immunofluorescence

Fig. 6.2 Nasal epithelial cells from an uninfected control calf, labelled for BHV1 antigen by immunofluorescence.

The cells are discernible only by the orange fluorescence of the counterstain.

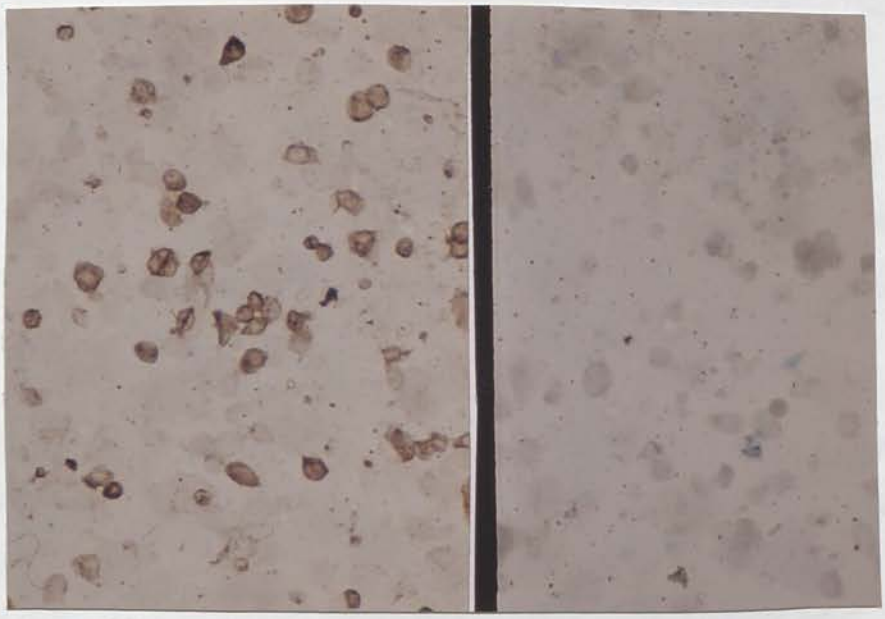


Fig. 6.3 Nasal epithelial cells from a BHV1 experimentally infected calf labelled by immunoperoxidase for BHV1 (L) and BVDV (R) antigens

ing pretreatments on the labelling of viral antigen was investigated. 0.5% hydrogen peroxide in aqueous solution produced gas bubbles which disrupted the smears from the slide. 0.5% hydrogen peroxide in methanol (10 minutes) markedly reduced the positive labelling of viral antigen and was therefore considered unsatisfactory. 0.1% phenylhydrazine (1 hour at 37°C and pH 7.0) did not impair the antigen labelling and has been shown to block effectively endogenous peroxidase activity (Miry and others, 1983). This finding was supported by other work by the author (unpublished observations) on frozen and paraffin sections of bovine tissues.

Following the successful results with the cell labelling technique in the experimental animals, and encouraging results with preliminary studies on field samples received at the CVL, it was decided to make the immunofluorescence technique widely available through the Veterinary Investigation Service in England and Wales. The programme consisted of 5 parts:-

(i) provision of fluorescent antibody conjugate for the direct labelling of smears and tissues from suspect IBR cases. The antiserum was prepared in a colostrum deprived calf, and conjugated to FITC, in bulk quantities. The conjugate was freeze dried in 2 ml aliquots for distribution to the diagnostic laboratories. Freeze drying was found to cause only a slight drop in optimum working dilution.

(ii) training of staff from every VI centre, so that they could distinguish specific and non-specific fluorescence, recognize characteristic features of the fluorescence for IBR (and other viruses), and learn the appropriate technical procedures.

(iii) provision of suitable equipment (i.e. fluorescence microscopes) for each centre.

(iv) monitoring of performance through a quality assessment scheme.

(v) recording of results through a voluntary reporting scheme, the data being collated by the author in a Prime Information account on the CVL computer.

The recording scheme began during 1984 and was fully operational in time to provide a full year's data for 1985. The results for IBR are shown in table 6.4. The figures shown are for disease episodes on individual farms. Data was also collected on an individual animal basis which gave diagnostic rates for IBR of 10.0% for live animals with respiratory disease (i.e. nasal cell smears, 336/3366 cases), and 4.8% for dead cases of respiratory disease (i.e. sections or smears of trachea and lung, 51/1067 cases). In addition, of 138 aborted fetuses examined for BHV1 antigen by immunofluorescence, six (4.3%) were reported positive (5/134 outbreaks). There were insufficient positives for a meaningful monthly analysis of abortion results although there was a marked seasonal trend in the number of fetuses examined, most being from May-November, with the peak in August.

A chi-squared analysis on the monthly figures from respiratory disease outbreaks showed a significant seasonal trend ($P<0.001$) both in numbers of outbreaks examined, and the proportion of these attributed to BHV1 infection (Table 6.4). It should be emphasised that immunofluorescence is not the sole diagnostic test used to confirm IBR in many outbreaks. A more comprehensive account of confirmed IBR outbreaks has been given in chapter 3. The purpose of the present

analysis is to assess the value of immunofluorescence as a diagnostic tool.

For quality assessment, VI centres were sent coded acetone-fixed nasal cell smears from the experimental animals to label by immunofluorescence. A summary of the overall performance is shown in table 6.5, although this fails to reveal that some individual centres consistently achieved 100% success, whilst others have had technical problems on particular occasions although generally performing well. The overall sensitivity of the VI centres, compared with CVL, was 81% (95% confidence limits $\pm$ 8%) and specificity was 88% ($\pm$ 10%).

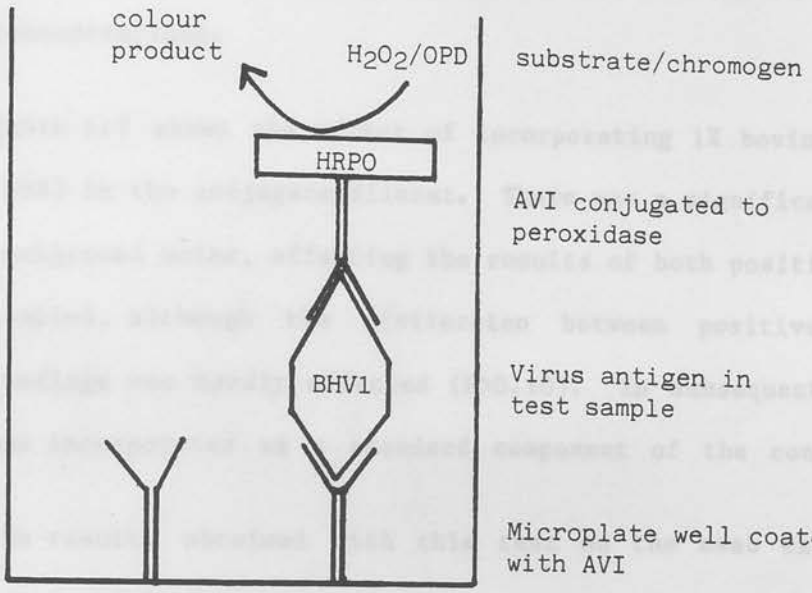
ELISA FOR ANTIGEN DETECTION

An antigen-capture ELISA was developed for the detection of BHV1 antigen in the supernatants of swab transport media. The influence of a number of technical variations on the test results was assessed, including the use of the avidin-biotin system to amplify the response. Microtitre immunoassay plates were coated overnight at 4°C with 100 µl per well of IgG from the gnotobiotic calf antiserum to BHV1 diluted 4µg/ml in carbonate coating buffer. The plates were washed three times in PBST, then dried and stored at 4°C to await testing. Undiluted samples were added for 2 hours at 37°C, then the plates washed before addition of the detection steps for bound antigen.

In the simple sandwich (SS) assay, peroxidase conjugated IgG from the gnotobiotic antiviral serum was added at a dilution in ELISA diluent of 1/1000 from 50% glycerolised stock conjugate. The optimal concentrations of coating and conjugate layers were determined by chequerboard titrations with a known positive sample (cell culture

harvest of the virus). The working dilutions were chosen to optimise both the signal/noise ratio and the absolute difference in OD reading between positive and negative samples. The chromogenic substrate was OPD and the colour reaction stopped after 15 minutes by the addition of 25 µl per well 2.5M sulphuric acid. The steps used in the SS assay are shown diagramatically in fig. 6.4.

The type of microtitre plate used had a marked effect on the results. Table 6.6 shows the comparison between plate types M129A and M129B (Dynatech Laboratories Ltd.) for a sample close to the lower limit of antigen detectability. M129B gave unacceptably high colour reactions in the negative sample wells and was not used further, even though it produced much higher ODs with the positive samples. Both these plates were of the rigid polystyrene type, and M129A plates were used for subsequent work (Tables 6.7-6.8) until flexible polyvinyl types became available. For the statistical analysis in table 6.6, the arithmetic difference in OD between the weak positive and the negative sample was calculated for each location in the chequerboard matrix. This was more useful than the ratio (positive: negative) which became very high, even with weak positive reactions, as the negative value approached zero. The test was analysed as a three-way anovar. The concentration of coating antibody did not significantly affect the discrimination between positive and negative, although at the higher levels it markedly increased the absolute OD values, giving an unacceptably high colour reaction with the negative sample (data not shown). Addition of 4% polethylene glycol (PEG, molecular weight 6000) to the conjugate diluent as suggested by Salonen and Vaheri (1981) appeared to increase slightly the OD of the positive sample without affecting the negative readings. This effect was not



AVI = gnotobiotic bovine anti-viral immunoglobulin
HRPO = horse radish peroxidase

Fig. 6.4 Schematic representation of the simple sandwich (SS) ELISA for BHV1 antigen detection

statistically significant and, after use in a few more trials, the addition of PEG to the diluent was discontinued. The only significant source of variation in table 6.6 was the concentration of conjugate used. The use of 1/1000 dilution of the conjugate for the test was selected on the basis of this and further tests at varying antigen concentrations.

Table 6.7 shows the effect of incorporating 1% bovine serum albumen (BSA) in the conjugate diluent. There was a significant reduction in background noise, affecting the results of both positive and negative samples, although the distinction between positive and negative readings was hardly affected ($P>0.10$). In subsequent tests, 1% BSA was incorporated as a standard component of the conjugate diluent.

The results obtained with this test on the swab extraction medium supernatants from experiment 4 are shown in table 6.8 and fig 6.5. The mean of the 30 results for the control (non-infected) calves was 0.087 with a standard deviation of 0.046, a standard error of 0.008 and upper tolerance limits (using one-tailed values for t) of 0.17 (at $P = 0.05$) and 0.24 (at $P = 0.001$). In interpreting the test these values were used to define positive (>0.24), negative (<0.17) and indeterminate results. It can be seen in table 6.8 that two samples from the noninfected calves fell into the inconclusive category. The infected calves were positive from day 2 to day 8 (2 calves) or to day 11 (1 calf).

A further assessment of the effects of microtitre plate characteristics on the results of the ELISA was made by comparing the Dynatech M129A type (rigid polystyrene) with Falcon 3912, Microtest III (Becton, Dickinson & Co) (flexible polyvinyl type). As shown in

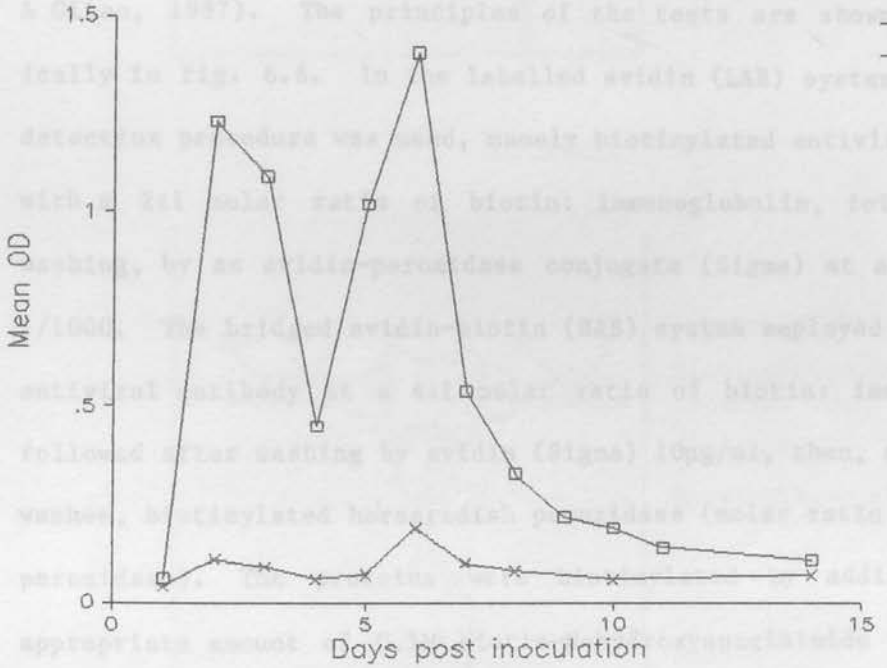
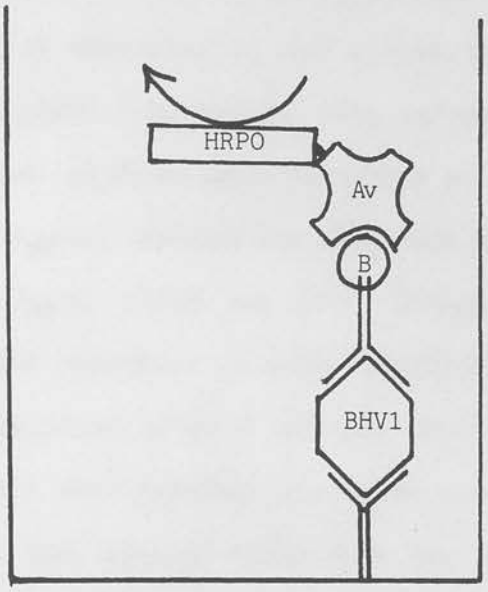


Fig. 6.5 Simple sandwich ELISA for BHV1 antigen. Mean OD values for nasal swab extracts from three infected and three control calves (experiment 4)

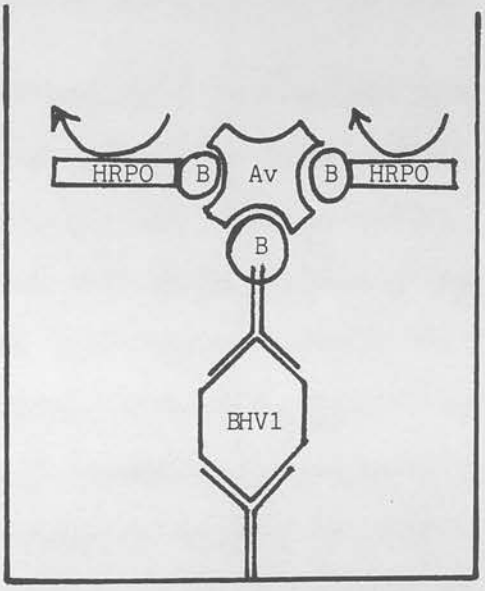
table 6.9 there was a small but significant rise in background OD values with negative samples on the polyvinyl plate, but this was considered on acceptable disadvantage, in view of the much greater, and highly significant ($P<0.001$), increase in positive OD values on the same plates. For subsequent work, the flexible polyvinyl plates were used.

The development of the amplified ELISA utilising the avidin-biotin interaction has been described fully elsewhere (Gitao, 1984; Edwards & Gitao, 1987). The principles of the tests are shown diagrammatically in fig. 6.6. In the labelled avidin (LAB) system a two stage detection procedure was used, namely biotinylated antiviral antiserum with a 2:1 molar ratio of biotin: immunoglobulin, followed, after washing, by an avidin-peroxidase conjugate (Sigma) at a dilution of 1/1000. The bridged avidin-biotin (BAB) system employed biotinylated antiviral antibody at a 4:1 molar ratio of biotin: immunoglobulin, followed after washing by avidin (Sigma) 10µg/ml, then, after further washes, biotinylated horseradish peroxidase (molar ratio 10:1 biotin: peroxidase). The proteins were biotinylated by addition of the appropriate amount of 0.1M biotin-N-hydroxysuccinimide (Sigma) dissolved in dimethylformamide to a 10 mg/ml solution of the protein in 0.1M sodium bicarbonate. After reacting for 1 hour at room temperature, unbound biotin was removed by dialysis against PBS pH 7.2.

As a further refinement on the three ELISA systems (SS, LAB and BAB) control wells were introduced, coated with bovine immunoglobulin lacking antibodies to BHV1. The test results were then calculated as (OD in test well - OD in control well). When calculated by this method, the 0.1% upper tolerance limit for negative samples (the negative/positive cut off point) became 0.10.



LAB assay



BAB assay

AVI = gnotobiotic bovine anti-viral immunoglobulin

Fig. 6.6 Schematic representation of the amplified ELISAs for BHV1 antigen detection: labelled avidin biotin (LAB) and bridged avidin biotin (BAB) systems

The sensitivity of the three ELISAs was compared by titrating, to the end-point of detection in each system, both density-gradient purified BHV1 and crude freeze-thaw cell culture harvest of BHV1. The SS could detect gradient-purified virus at a concentration equivalent to $10^{6.0}$ $TCID_{50}$/ml, whereas the amplified ELISAs had detection limits of $10^{4.5}$ $TCID_{50}$/ml (LAB) and $10^{4.2}$ $TCID_{50}$/ml (BAB), equivalent to 30- and 60-fold increases in assay sensitivity, and a detection rate for BAB of 5µg viral protein per test well (100µg/ml). The sensitivity enhancement when testing the crude viral harvest was approximately 10- (LAB) and 20-fold (BAB) over the SS method, as illustrated in fig. 6.7.

REVERSE PASSIVE HAEMAGGLUTINATION (RPHA)

Sheep red blood cells were washed, then treated with chymotrypsin as described by Cranage and others (1985). Anti-viral specific immunoglobulin was prepared from gnotobiotic calf antiserum to BHV1 using the caprylic acid method (Steinbuch and Audran, 1969). After dialysis against 0.9% sodium chloride the antibody was coupled to the enzyme treated erythrocytes using chromic chloride (Scott and others 1981). A 1% suspension of the coated cells was stabilised by glutaraldehyde treatment (Cranage and others, 1983) and kept at 4°C. The test was carried out by mixing 30µl of serial doubling dilutions of swab extraction media with equal volumes of 1% coated red cell suspension in a 96 well round-bottomed micro-titration plate. The haemagglutination reaction in each well was graded visually, after 2 hours at room temperature, on a scale from 0 (absent) to 4 (complete agglutination). The end point was taken as the highest dilution giving a reading of at least 2. The controls used for each sample

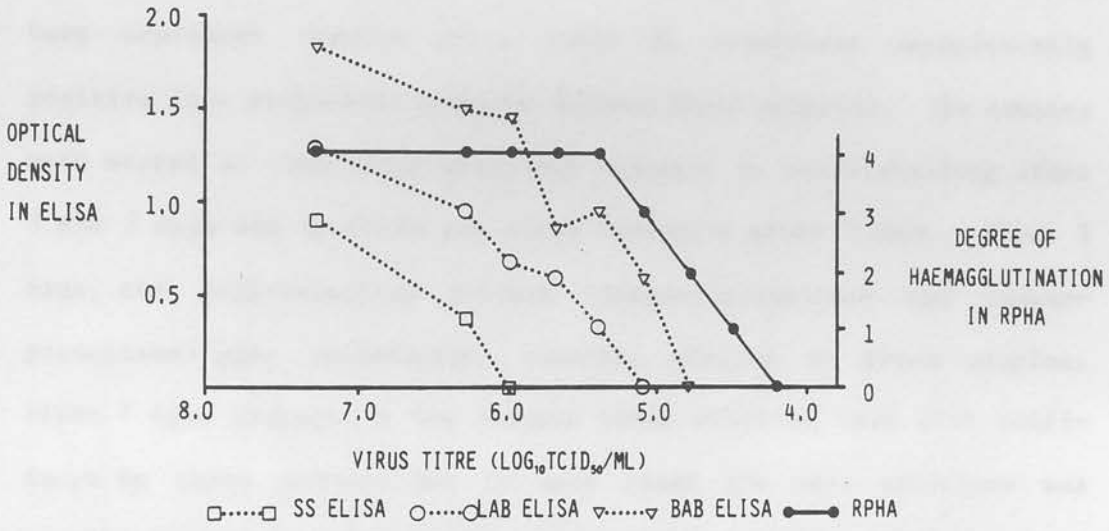


Fig. 6.7 Comparative sensitivities of three ELISAs and RPHA for the detection of BHV1 antigen in serial dilutions of cell culture fluid

were uncoated sheep erythrocytes, and sheep erythrocytes coated with antibody negative to BHV1.

The sensitivity of the RPHA on cell culture harvest of BHV1 was similar to the amplified BAB ELISA, as shown in fig. 6.7.

COMPARISONS OF ANTIGEN DETECTION METHODS

The extracts from the short nasal swabs in experiment 4 were tested for BHV1 antigen by immunofluorescent and immunoperoxidase labelling of cell smears and by SS ELISA. The comparison of the 3 methods in relation to virus isolation is shown in fig. 6.8. Only the solid bars represent results which would be considered unequivocally positive in a diagnostic assay on unknown field material. The samples were stored at room temperature and retested by cell-labelling after 3 and 7 days and by ELISA and virus isolation after 7 days. After 3 days, the cell-labelling systems (immunofluorescence and immunoperoxidase) gave satisfactory results, similar to fresh samples. After 7 days storage, a few samples could still be read with confidence by these methods but in most cases the cell structure was greatly disrupted, there were very few intact cells in the smears, and although fluorescence or coloured substrate were still detectable in the cell debris of positive samples a confident diagnosis could not be established. The effects of room temperature storage on the ELISA and virus isolation results are shown in fig. 6.9. The strong positives remained positive but there was a reduction in positive results for both tests from the eighth day after inoculation. Gross bacterial or fungal contamination of some of the samples was evident after the week at room temperature, but this did not appear to interfere with the results.

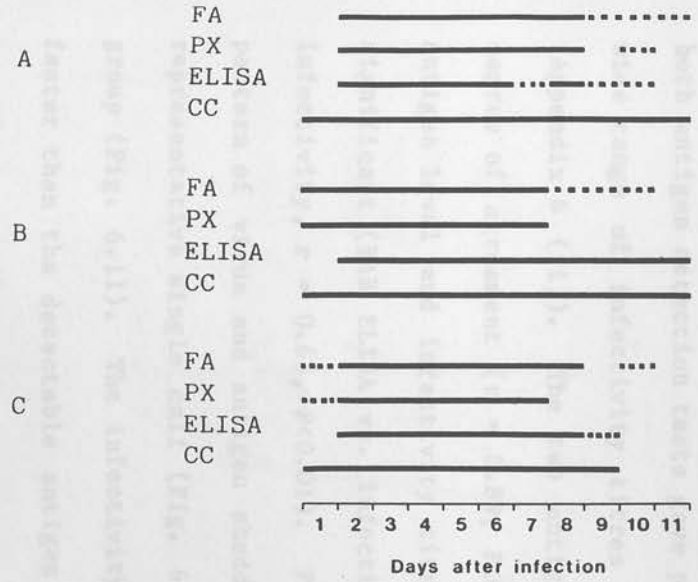
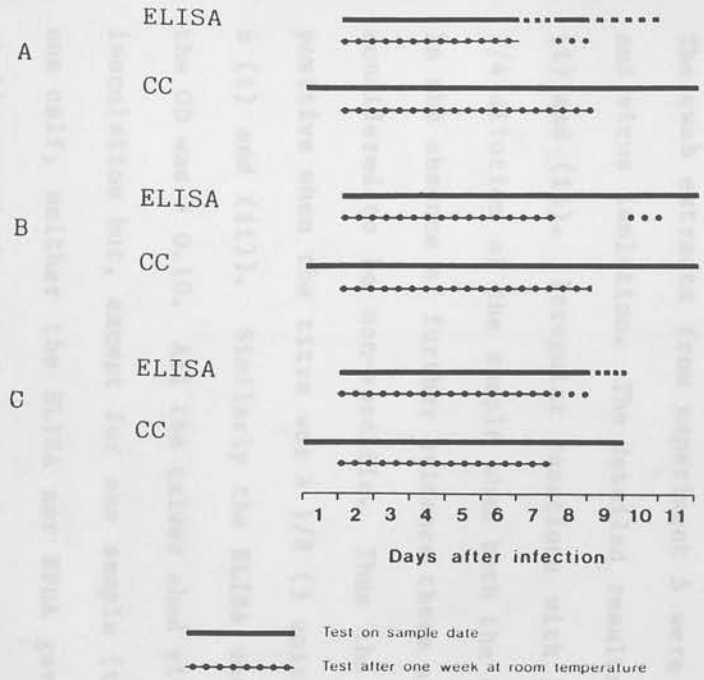


Fig. 6.8 Detection of BHV1 in nasal swab extracts from infected calves (A,B and C) by four methods: immunofluorescence (FA), immuno-peroxidase (PX) ELISA and cell culture (CC)



Broken bars indicate weak positive results

Fig. 6.9 ELISA and CC results from fig. 6.8 compared with the same tests after storing the samples for a week at room temperature

The swab extracts from experiment 5 were tested by BAB ELISA, RPHA and virus isolation. The detailed results are given in Appendices 6 (i) and (ii). Irregular reactions with RPHA occurred at up to the 1/4 dilution of the sample when both the other assays were negative. In the absence of further evidence these agglutination reactions were considered to be non-specific. Thus the RPHA was only classed as positive when the titre was $\geq$ 1/8 (3 units on the scale in appendix 6 (i) and (ii)). Similarly the ELISA was classed positive only if the OD was $\geq$ 0.10. All the calves shed virus following the challenge inoculation but, except for one sample (titre $10^{6.0}$ $TCID_{50}$/ml) from one calf, neither the ELISA nor RPHA gave a positive result after challenge of the vaccinated calves, the samples from which contained $\leq 10^{5.5}$ $TCID_{50}$/ml (Appendix 6 (i)).

In the unvaccinated calves undergoing primary challenge with BHV1, both antigen detection tests gave positive results for samples with a wide range of infectivity titres ($10^{3.5}$ to $10^{6.75}$ $TCID_{50}$ per ml) (Appendix 6 (ii)). The two antigen detection tests showed a high degree of agreement ($r = 0.89$, $P<0.01$) but the correlation between antigen level and infectivity titre was much poorer although still significant (BAB ELISA vs. infectivity, $r = 0.60$, $P<0.01$; RPHA vs. infectivity, $r = 0.65$, $P<0.01$). Figs. 6.10 and 6.11 illustrate the pattern of virus and antigen shedding in the nasal secretions of a representative single calf (Fig. 6.10) and the mean values for the group (Fig. 6.11). The infectivity titre of virus appeared to rise faster than the detectable antigen level in the first 3 days after inoculation. Thereafter the curves for the antigen detection tests follow that for the infectivity titre, except that infectious virus remained detectable at a moderate to low level ($\leq 10^{5.25}$ $TCID_{50}$/ml)

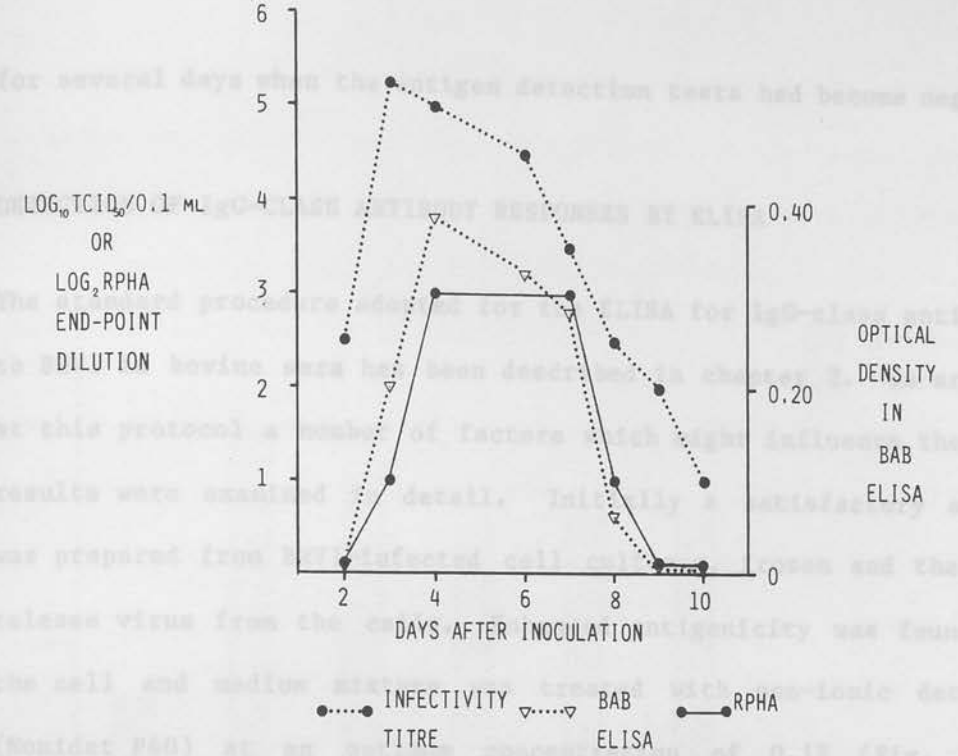


Fig. 6.10 Comparison of virus isolation, amplified ELISA and RPHA for the detection of BHV1 in nasal swab extracts from a single calf

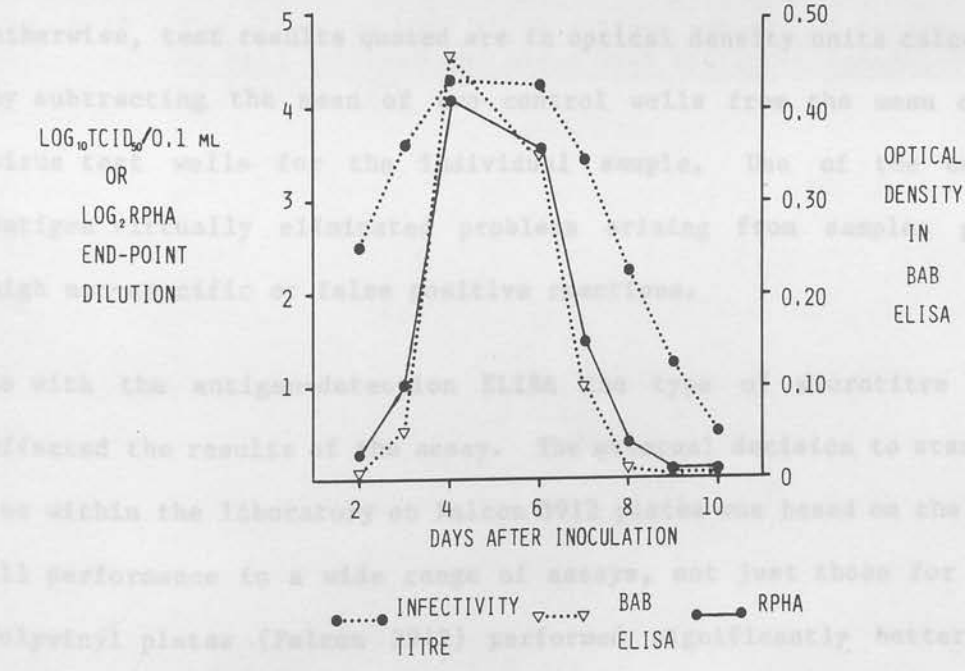


Fig. 6.11 Group mean results of 13 calves (experiment 5) for BHV1 detection in nasal swab extracts using cell culture, amplified ELISA and RPHA methods.

for several days when the antigen detection tests had become negative.

DETECTION OF IgG-CLASS ANTIBODY RESPONSES BY ELISA

The standard procedure adopted for the ELISA for IgG-class antibodies to BHV1 in bovine sera has been described in chapter 2. In arriving at this protocol a number of factors which might influence the assay results were examined in detail. Initially a satisfactory antigen was prepared from BHV1-infected cell cultures, frozen and thawed to release virus from the cells. Enhanced antigenicity was found when the cell and medium mixture was treated with non-ionic detergent (Nonidet P40) at an optimum concentration of 0.1% (Fig. 6.12). Subsequently a more potent antigen was prepared by omitting the freeze-thaw step, discarding the cell culture medium and treating the cellular fraction with 0.5% Nonidet P40. In all cases a control antigen was made from non-infected cell cultures. Unless stated otherwise, test results quoted are in optical density units calculated by subtracting the mean of two control wells from the mean of two virus test wells for the individual sample. Use of the control antigen virtually eliminated problems arising from samples giving high non-specific or false positive reactions.

As with the antigen-detection ELISA the type of microtitre plate affected the results of the assay. The eventual decision to standardise within the laboratory on Falcon 3912 plates was based on the overall performance in a wide range of assays, not just those for BHV1. Polyvinyl plates (Falcon 3912) performed significantly better than polystyrene (Dynatech M129A) as shown in table 6.10. This table also shows the effect of different storage procedures on the potency of antigen-coated plates. Storage in the wet state (i.e. with antigen

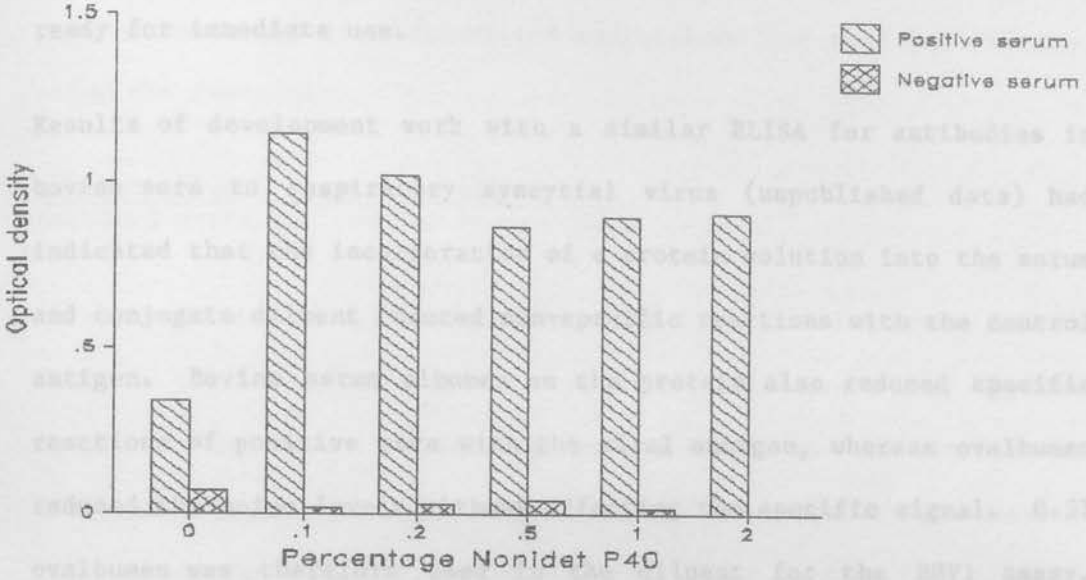


Fig. 6.12 The effect on BHV1 ELISA antigen potency of treatment of cell cultures and media with different concentrations of nonidet P40

solution still in the wells) at either 4°C or -20°C appeared to preserve the antigenicity better although the effects were not significant in this experiment. For practical reasons it was often more convenient to wash and dry a batch of plates so that they were ready for immediate use.

Results of development work with a similar ELISA for antibodies in bovine sera to respiratory syncytial virus (unpublished data) had indicated that the incorporation of a protein solution into the serum and conjugate diluent reduced non-specific reactions with the control antigen. Bovine serum albumen as the protein also reduced specific reactions of positive sera with the viral antigen, whereas ovalbumen reduced the noise levels without affecting the specific signal. 0.5% ovalbumen was therefore used in the diluent for the BHV1 assay.

In an attempt to further reduce background noise levels, extraction of the BHV1 and control antigens with an organic fluorocarbon solvent (Arklone, ICI), with or without n-butanol, was evaluated (Nandapalan and others, 1984). In addition, post-coating of the plates with 2% gelatin in PBS pH 7.2 was tried, to block any unoccupied binding sites on the plastic. The results are in table 6.11. Gelatin treatment had no significant effect, suggesting that any non-specific colour reactions were not due to unoccupied protein binding sites on the plastic. Arklone extraction significantly increased ($P<0.01$) the binding to the control antigen (columns C in Table 6.11) and decreased the specific binding with viral antigen. Further treatment of the antigen, by n-butanol precipitation of the protein and redispersal, almost completely destroyed its antigenicity. None of these methods was pursued further.

The effect of variation between wells on the plate was studied by coating two plates with virus antigen in all wells, and adding aliquots of a single dilution (1/100) of a positive serum to all wells for the test. Two-way analyses of variance were carried out and the results expressed as standardized differences for rows and columns, using the formula:-

$$\frac{\text{Row (or column) mean - overall mean}}{\text{Standard error for row (or column) mean}}$$

As shown in table 6.12, there were significant differences between wells on the same plate and the most marked differences were observed in the end columns. Low and high values tended to occur in adjacent rows and columns, giving a clustering effect. Differences between columns were more highly significant than those between rows.

For the subsequent tests the microtitre plates were sub-divided into 24 blocks of 4 wells. Each block provided 2 readings for the test serum dilution with viral antigen (T1 and T2) and two with control antigen (C1 and C2). A set of six pairs of acute and convalescent sera from an IBR outbreak in Northamptonshire were tested by ELISA (a) by titrating the sera in a two-fold dilution series from 1/50 to 1/6400, (b) by testing at a single serum dilution of 1/100. In the latter test, eight replicate blocks were used for each serum, with four 4-well blocks allocated at random on each of two plates. Analysis of variance was used to estimate the variation between and within blocks, and to determine the least significant difference in OD (between acute and convalescent sera from the same animal) necessary to establish a diagnostic rise in antibody level.

The log dilution - response curves for ten of the twelve sera were

approximately linear over the dilution range 1/50 to 1/3200 or 1/50 to 1/6400 (Table 6.13 and Fig. 6.13). Except for pair 3, there was a fourfold or greater rise in VN titre between the acute (A) and convalescent (C) samples and there was an increase in slope of the ELISA curve from A to C. The change in ELISA antibody level from acute to convalescent was measured in three ways (Table 6.13): the estimated end point titres for each serum (i.e. the dilution at which the OD would be 0.10), the ratio OD_C/OD_A and the arithmetic difference OD_C-OD_A at a given serum dilution. The ratio method was subject to high bias when the acute OD value was low. The best discrimination between A and C was achieved with the arithmetic difference in OD at a dilution of 1/100.

The results of the second part of this study, in which 8 replicate blocks were tested for each of the 12 sera at 1/100 dilution, are shown in table 6.14. The mean differences between sera A and C for each animal were significant ($P<0.001$) except for pair 3, which did not differ significantly in any of the tests. There was again evidence of clustering of OD values on the plate, in that the variance ratios (F) of the difference between blocks of the same serum in different areas of the plate, to that between duplicates within blocks was significant (Table 6.15).

In the above experiment, all the replicate tests for any one serum were made from one bulk dilution of the sample. Thus the variation observed was due to plate effects. In order to assess the total variation within the whole assay procedure tests were carried out on three sera of which a bulk supply was available, with the intention that they could henceforth be used as standards for the assay. The sera were collected from three calves, one of which (reference SA37)

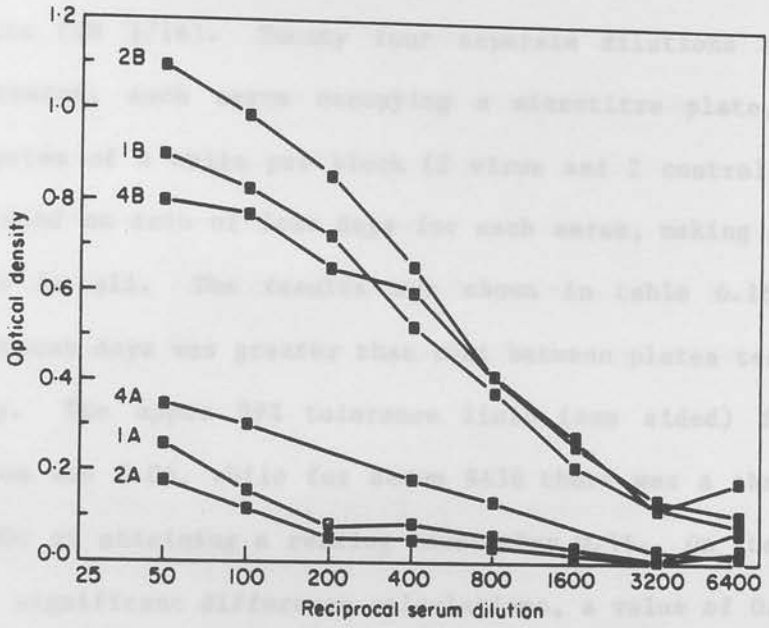


Fig. 6.13 The log dose - response in ELISA for three pairs of acute (A) and convalescent (B) sera from a natural outbreak of IBR

was seronegative to BHV1, one (SA38) had been immunised with intranasal BHV1 vaccine (Tracherine, Smith Kline Animal Health) and had a low antibody titre to the virus (VN titre 1/5), and the third (SA39) had been infected with virulent BHV1 and had a typical convalescent antibody titre (VN 1/16). Twenty four separate dilutions of each serum were tested, each serum occupying a microtitre plate, again using the system of 4 wells per block (2 virus and 2 control). Two plates were used on each of four days for each serum, making a total of 24 plates in all. The results are shown in table 6.16. The variation between days was greater than that between plates tested on the same day. The upper 99% tolerance limit (one sided) for the negative serum was 0.06, while for serum SA38 there was a chance of only 1 in 1000 of obtaining a reading lower than 0.16. On the basis of the least significant difference calculations, a value of 0.15 was set as the change in OD indicative of a significantly rising antibody response between acute and convalescent sera from the same animal.

Paired sera from 105 cattle with respiratory disease, submitted to the laboratory for diagnostic purposes, were tested using a rise in OD of 0.15 as the criterion for a positive diagnosis, and comparing it with the conventional fourfold rise in VN titre. The two tests agreed in 103 of the serum pairs (98%), of which 87 were positive (rising titre) and 16 negative (no change in titre). The remaining two pairs showed no change by VN, but a rise in OD in one case of just 0.15, and in the other 0.46. The latter animal was from a group experiencing clinical IBR in which other animals showed a rising titre by both tests. To give a margin of safety the OD value has been raised to 0.2 for routine diagnostic use.

As well as diagnostic assays for clinical cases, serological tests for BHV1 are also used for screening purposes for animals prior to

entry to sero-negative herds such as bull studs or for export certification. A large group of such sera, of known VN status, were tested by the ELISA in order to establish a positive/negative cut off value. Six hundred and fifty two sera were from herds known to have been free of BHV1 infection for many years and therefore true negatives. Eighty eight were definitely sero-positive by VN, and 181 were weak positives giving low titre reactions (<10) in the VN test. 119 of the latter group were subsequently retested by ELISA giving a total of 300 readings for the weak positive group. The results are summarised in Table 6.17. All the sero-negative samples gave results <0.10.

The sero-positive group were all >0.10 with a mean OD of 1.21 (standard error +/- 0.045). There was a less than 1 in 1000 chance of one of this group giving a result <0.10. The weak positive group could be divided into two populations (Table 6.17) based on VN titres of <4 and 4 and above, with considerable overlap in OD values. A few of this group gave high OD readings (confirmed on retest) suggesting erroneous VN results (which were not repeated). 112 of the weak positives gave ODs <0.10, at least some of which may have been false positive results by VN (nonspecific neutralization). Thenceforth a negative/positive cut-off value of 0.10 was used, with the acceptance that a low number of sera would inevitably be misclassified, by this or any other system. The sensitivity of the assay for screening purposes was found to be slightly higher when sera were tested at 1/50 dilution, rather than the 1/100 which gave the best discrimination between acute and convalescent sera for diagnosis of clinical disease.

DETECTION OF IgM-CLASS ANTIBODIES BY ELISA

As described in Chapters 1 and 5 IgM-class antibodies to BHV1 appear early in the course of infection and are of short duration. Their detection should provide an adjunct to the rapid diagnosis of IBR, and could obviate the need for collection of convalescent serum samples in many cases. The immunoglobulin sub-class specific ELISA was described in Chapter 2, using viral antigen coated plates, heavy chain specific rabbit antisera to bovine immunoglobulins, and protein A enzyme conjugate. Conjugate controls, omitting the intermediate rabbit serum were used for all test samples to detect any binding of the protein A to bovine IgG. A goat anti-rabbit IgG peroxidase conjugate (Miles Scientific) was also assessed in the early trials, but was not pursued as it gave poorer signal/noise ratios.

A standard serum for the IgM assays was prepared by killing and bleeding out a calf, 10 days after the intranasal inoculation of 10^7 $TCID_{50}$ BHV1. The assay was subsequently standardized by carrying out a titration of this serum on each occasion that a test was performed.

To establish the immunoglobulin class specificity of the assays, purified IgG1 and IgG2 were prepared from a high titre bovine antiserum to BHV1 by the methods of Fey and others (1976) for IgG1 and Butler (1983) for IgG2. IgM was purified from the standard serum containing high BHV1-specific IgM, by the method of Hudson and Hay (1980), using affinity chromatography with protamine sulphate followed by gel filtration on Sepharose 6B (Pharmacia).

The results of the class specific assays for BHV1 antibodies in the resultant fractions are shown in Table 6.18. The protein A conjugate bound weakly to bovine IgG2 only. In practice this was found not to

cause any difficulties with the test samples, provided conjugate controls were used.

The possibility that the fall in IgM in week 3 after inoculation (Chapter 5) was an artefact caused by competition for antigenic sites by IgG, which was rising at this time, was investigated by testing protamine sulphate purified IgM (Hudson and Hay, 1980) fractions of serial samplings from 2 of the animals in experiment 1C. Removal of most of the IgG from the samples did not greatly affect the shape of the IgM response curve, as compared with the whole serum assay. The same fall in IgM was observed during the third week after inoculation (Fig. 6.14).

Paired acute and convalescent sera from natural cases of respiratory disease were obtained from the laboratory's diagnostic section. They were classified into three categories according to the results obtained with the VN test for BHV1 antibodies:

a. both samples seronegative to BHV1;
b. evidence of active BHV1 infection, fourfold or greater rise in VN titre between acute and convalescent sample;
c. both samples seropositive, VN titre level or falling;

The results from cattle (group a) with no evidence of BHV1 infection, indicated that there was a considerable variation in background colour reaction, with some animals giving much higher ODs in the IgM assay (with both control and virus antigens) than the IgG assays. The colour difference between virus and control antigen was also frequently as high as 0.25 OD units, so that a positive result for IgM could only be accepted for samples with an OD difference >0.25. The spread of the results from field sera is shown in figure 6.15, while

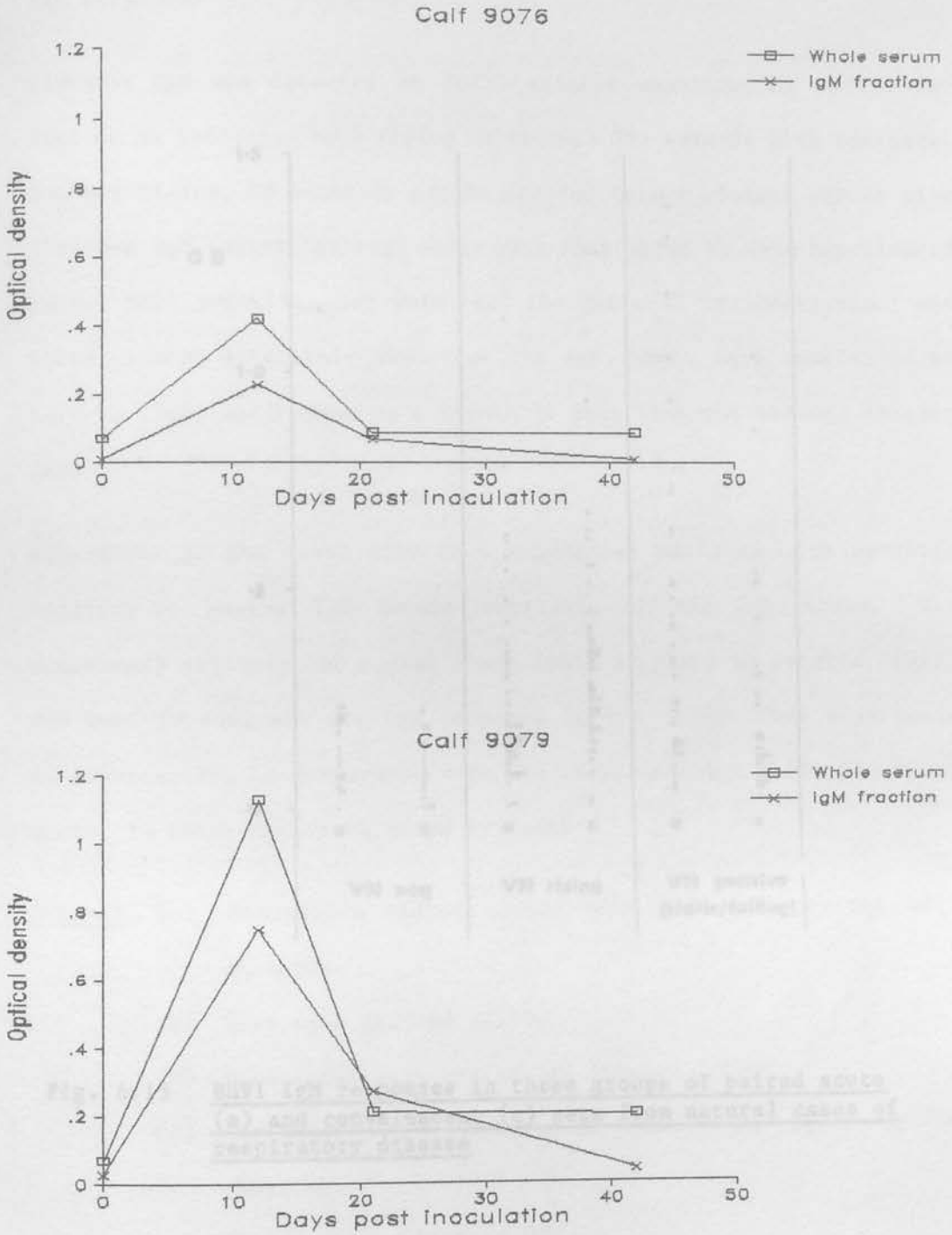


Fig. 6.14 Comparison for two calves of IgM-specific ELISA responses on whole sera diluted 1/100 and on purified IgM fractions of the sera diluted 1/20

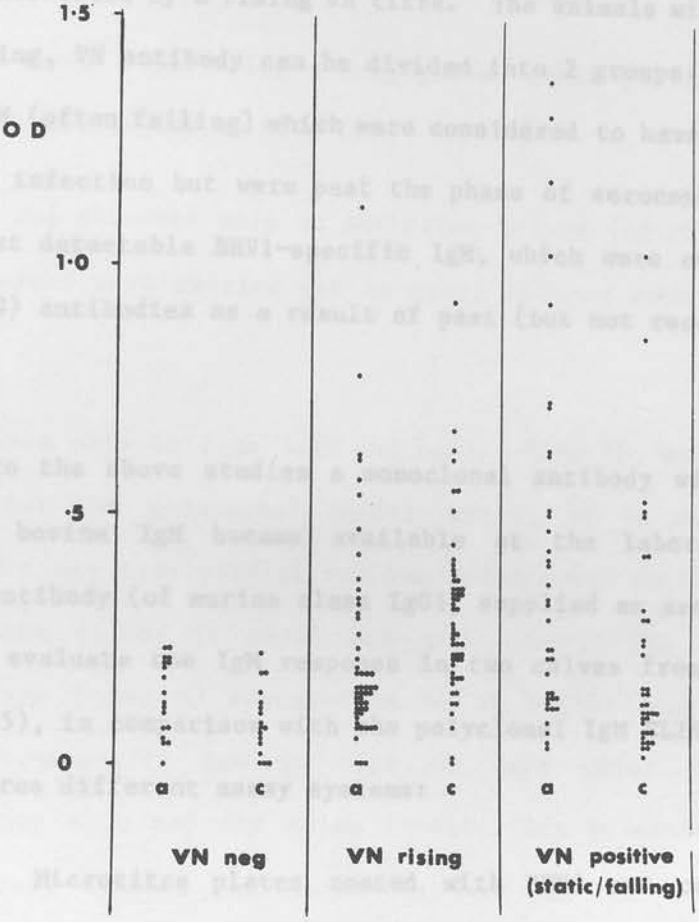


Fig. 6.15 BHV1 IgM responses in three groups of paired acute (a) and convalescent (c) sera from natural cases of respiratory disease

in table 6.19 they are grouped according to the dynamic state of the IgM response.

Elevated IgM was detected in 30/53 animals experiencing active infection as indicated by a rising VN titre. The animals with positive, but not rising, VN antibody can be divided into 2 groups: those with elevated IgM (often falling) which were considered to have experienced recent BHV1 infection but were past the phase of seroconversion; and those without detectable BHV1-specific IgM, which were considered to have VN (IgG) antibodies as a result of past (but not recent) infection.

Subsequent to the above studies a monoclonal antibody with specific activity to bovine IgM became available at the laboratory. The monoclonal antibody (of murine class IgG1, supplied as ascitic fluid) was used to evaluate the IgM response in two calves from experiment 1C (Chapter 5), in comparison with the polyclonal IgM ELISA described above, in three different assay systems:

Assay 1
(a) Microtitre plates coated with BHV1 and control cell antigens.
(b) Test sera diluted 1/100.
(c) Monoclonal anti bovine IgM diluted 1/2000
(d) Peroxidase conjugated goat anti-mouse IgG1, 1/2000 (Nordic).
(e) Chromogenic substrate OPD.

Assay 2
(a) Microtitre plates coated with monoclonal anti-bovine IgM, 1/2000.
(b) Test sera diluted 1/100.

(c) BHV1 partially purified antigen (prepared by PEG precipitation as described in Chapter 4).

(d) Peroxidase conjugated bovine antiserum to BHV1, 1/1000.

(e) Chromogenic substrate OPD.

Assay 3 As assay 2 but using a conjugated rabbit antiserum to BHV1 (diluted 1/500) at step (d).

All buffers and diluents were as described before for the IgG ELISA, and plate washes were carried out as usual between each step of the assays.

The results are show in figs 6.16 and 6.17. When the monoclonal was substituted for the polyclonal rabbit serum, as in assay 1, the response curve was very similar but the noise level in the post-convalescent phase (after 21 days) was much lower. The IgM capture systems (assays 2 and 3) appeared to be of higher sensitivity and detected virus-specific IgM up till 28 days after inoculation. Thereafter they also had low noise levels. The potential of these monoclonal assay systems for diagnostic work has not, as yet, been further explored.

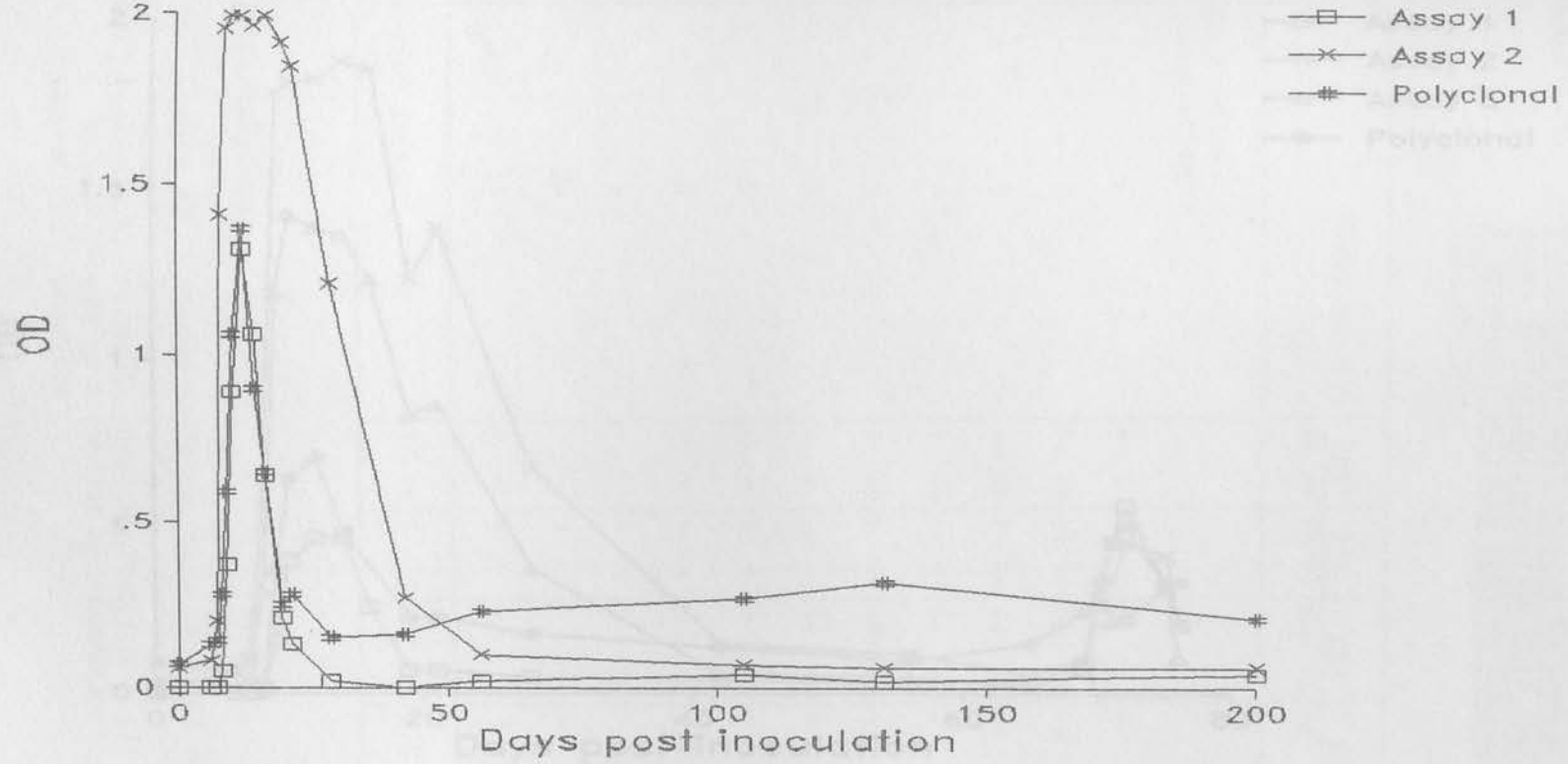


Fig. 6.16 Comparison of monoclonal IgM-capture (assay 2) with indirect antigen-coated plate systems (monoclonal assay 1 and polyclonal assay) on serum samples from a calf in experiment 1C, inoculated with BHV1 strain ED4

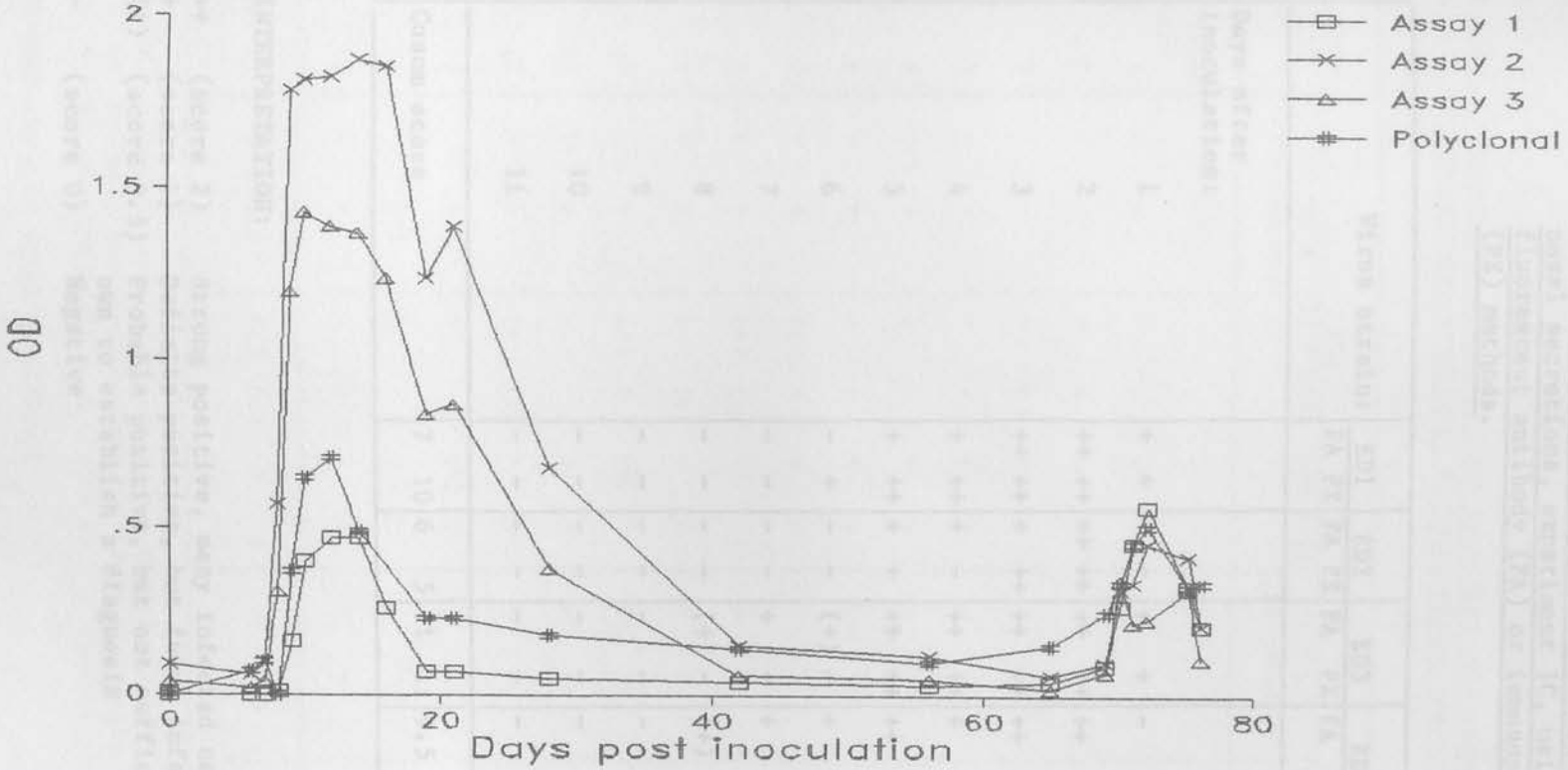


Fig. 6.17 BHV1 specific serum IgM response of a calf in experiment 1C which was inoculated with virus strain ED5, then challenged intravenously 65 days later with strain ED2: comparison of three monoclonal assays and a polyclonal method as described in the text

TABLE 6.1 Detection of BHV1 antigen in washed cell smears from nasal secretions, experiment 1C, using direct fluorescent antibody (FA) or immunoperoxidase (PX) methods.

Virus strain:	ED1		ED2		ED3		ED4		ED5		ED6	
	FA	PX	FA	PX	FA	PX	FA	PX	FA	PX	FA	PX
Days after inoculation:												
1	+	+	+	-	+	+	-	-	-	+	-	-
2	++	++	++	++	++	++	++	++	+	+	+	+
3	++	++	+	++	++	++	++	++	++	++	+	-
4	+	++	+	-	++	++	+	++	+	++	+	+
5	+	++	+	+	++	++	++	++	++	++	+	+
6	-	+	-	-	(+)	+	+	++	(+)	+	-	+
7	-	-	-	-	+	+	+	+	-	-	-	-
8	-	-	-	-	(+)	-	(+)	(+)	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
Cusum score	7	10	6	5	11	11	9.5	11.5	6.5	9	4	4

INTERPRETATION:

++	(score 2)	Strong positive, many infected cells in smear
+	(score 1)	Definite positive, but fewer infected cells
(+)	(score 0.5)	Probable positive, but not sufficient on its own to establish a diagnosis
-	(score 0)	Negative

PAIRED COMPARISON OF FA AND PX TESTS:

Mean difference in cusum score (PX-FA) = 1.083
Standard deviation = 1.625
Student's t (5 d.f.) = 1.63 (P >0.10)

TABLE 6.2 Detection of BHV1 antigen in washed cell smears from nasal secretions, experiment 2, using direct immunofluorescence.

Virus:	ED1						ED2					
Calf:	9366 (in-contacts)	9414 (in-contacts)	9378	9408	9409	9416	9370 (in-contacts)	9411 (in-contacts)	9406	9407	9412	9415
Days after inoculation: 1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	+	+	+	+	-	-	-	-	(+)	-
3	-	-	+	+	+	+	-	-	+	+	+	-
4	-	-	+	+	+	+	-	-	(+)	+	+	-
5	(+)	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	(+)	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	(+)	(+)	+	(+)	+	+	(+)	(+)	+	(+)
9	+	+	+	(+)	+	+	+	+	(+)	-	(+)	-
10	(+)	+	(+)	+	(+)	-	(+)	(+)	-	-	-	-
11	(+)	+	-	-	-	-	+	(+)	-	-	-	-
12	-	+	(+)	(+)	-	(+)	-	+	-	-	-	(+)
13	(+)	-	-	-	(+)	-	-	(+)	-	-	-	-
14	-	(+)	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
Cusum score	6	8.5	8.5	8.5	9	8	6	7.5	5.5	5.5	7	4

NOTES: + Definite positive (score 1, not graded further)
(+) Probable positive, but not of itself diagnostic (score 0.5)
- No positive cells seen (score 0)

PAIRED COMPARISON OF VIRUS STRAINS:

Mean difference in cusum score (ED1-ED2) = 2.17
Standard deviation = 1.47
Student's t (5 d.f.) = 3.606 ($P < 0.05$)

TABLE 6.3 Proportion(%) of virus-positive cells in washed cell smears from nasal secretions, experiment 4, using direct immunoperoxidase.

	Calf:	1	2	3
Days after inoculation:	1	-	<1	<1
	2	32	44	29
	3	63	74	37
	4	48	62	22
	5	16	14	8
	6	4	12	11
	7	2	<1	4
	8	13	-	-
	9	-	-	-
	10	<1	-	-
	11	-	-	-

TABLE 6.4 Summary of bovine respiratory disease outbreaks investigated by VI centres in 1985, in which immunofluorescent examinations for BHV1 antigen were carried out.

Month	No. of outbreaks	No. (and %) positive	Expected No. positive at a diagnosis rate of 13.5%
1	365	74 (20.3)	49
2	251	47 (18.7)	34
3	252	47 (18.7)	34
4	209	26 (12.4)	28
5	134	6 (4.5)	18
6	123	11 (8.9)	17
7	106	10 (9.4)	14
8	78	4 (5.1)	11
9	122	14 (11.5)	16
10	237	25 (10.5)	32
11	371	43 (11.6)	50
12	278	34 (12.2)	38
1985 Total	2526	341 (13.5)	
χ^2	513.4 (P <0.001)	41.16 (P <0.001)	

TABLE 6.5. Summary of VI centre performance in 31 coded quality assessment trials.

Year	Correct positives (%)	Correct negatives (%)
1982	30/39 (77%)	26/32 (81%)
1983	37/43 (86%)	23/27 (85%)
1984	56/69 (81%)	29/30 (97%)
TOTAL	123/151 (81%)	79/89 (88%)

TABLE 6.6 Comparison of two types of polystyrene plate, and use of polyethylene glycol (PEG) to enhance antigen antibody interactions, in the ELISA for antigen detection.

Plate type:	M129A				M129B			
4% PEG added:	-	-	+	+	-	-	+	+
BHV1 in sample:	+	-	+	-	+	-	+	-
Conjugate dilution								
1/500	.25	.08	.34	.04	.93	.65	.80	.64
1/1000	.16	.03	.18	.02	.38	.43	.74	.39
1/2000	.05	.02	.11	.01	.32	.20	.41	.20
1/4000	.04	.05	.04	.02	.48	.13	.35	.13

NOTES: 1. Results are given in OD units

2. Test samples were infected/non-infected cell culture harvests diluted 1/50 in PBS. This was taken as close to the virus detection limit for the assay and therefore a critical test of sensitivity.

3. The results are extracted, for clarity, from the full chequerboard assay of coating antibody level and conjugate dilution. The results shown are for the optimum coating level (4µg/ml).

Continued ...

TABLE 6.6 (Contd)

STATISTICAL ANALYSIS FOR PLATE M129A

Tabulated values are (OD positive sample - OD negative sample) x 100

Coating antibody: (µg/ml)	Without PEG						PEG added					
	32	16	8	4	2	1	32	16	8	4	2	1
Conjugate dilution												
1/500	4	26	25	17	13	12	57	27	32	30	20	17
1/1000	0	9	10	13	8	6	17	6	14	16	12	10
1/2000	2	2	5	3	3	3	8	8	9	10	6	4
1/4000	0	1	3	-1	4	-2	-3	4	-1	2	5	3

ANOVAR

Source of variation	d.f.	S.S.	M.S.	F ratios	
(a) PEG added or not	1	450	450	a/c=4.5(NS)	a/e=5.92(NS)
(b) Conjugate dilution	3	3323	1108	b/c=11.08(*)	b/f=41.04(***)
(c) PEG x conjugate	3	299	100	c/g=2.13(NS)	
(d) Coating antibody level	5	154	31	d/e=0.41(NS)	d/f=1.15(NS)
(e) PEG x coating	5	378	76	e/g=1.62(NS)	
(f) Conjugate x coating	15	404	27	f/g=0.57(NS)	
(g) Conjugate x coating x PEG	15	709	47		
TOTAL	47	5717			

NS = not significant (P>0.05)
* = P<0.05
*** = P<0.001

TABLE 6.7 The effect on non-specific background readings in the antigen detection ELISA, of 1% bovine serum albumen in the conjugate diluent.

		Sample Dilution				
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
	Sample					
Without BSA	C	.12	.09	.11	.11	.11
	I	>2.00	1.01	.16	.10	.13
Difference	(I - C)	>1.88	.92	.05	-.01	.02
With BSA	C	.12	.05	.05	.03	.04
	I	>2.00	.99	.11	.06	.05
Difference	(I - C)	>1.88	.94	.06	.03	.01

NOTES: Sample C = non-infected cell culture harvest
Sample I = BHV1-infected cell culture harvest
Conjugate diluent incorporated 4% PEG
Results shown are OD values, means of 2 replicates per sample.
Plate type was Dynatech M129A.

STATISTICAL ANALYSIS

Wilcoxon signed rank sum test for the comparison of OD values with BSA treatment vs. no treatment: $n = 8$; t, $T = 0, 36$; $P<0.05$

Mann-Whitney test for the comparison of (I - C) values with BSA teatment vs. no treatment: $n_1 = n_2 = 5$; $U = 10.5$; $P>0.10$
(note, insufficient values for a Wilcoxon test).

TABLE 6.8 Antigen-detection ELISA results (OD values) on swab supernatants from experiment 4.

Treatment:	BHV1 infected			Control		
Calf:	1	2	3	C1	C2	C3
Days after inoculation:						
1	.06	.06	.06	.05	.03	.04
2	.84	1.62	1.22	.08	.17	.08
3	1.05	1.54	.67	.09	.07	.10
4	.42	.43	.50	.05	.05	.09
5	.74	1.13	1.19	.09	.06	.07
6	1.07	1.41	1.76	.16	.16	.24
7	.22	.55	.85	.11	.07	.12
8	.33	.30	.37	.06	.06	.13
9	.20	.27	.19	ND	ND	ND
10	.17	.25	.14	.06	.07	.05
11	.10	.25	.06	ND	ND	ND
14	.12	.14	.06	.07	.07	.06

NOTE: ND = no sample taken.
Plate type was Dynatech M129A

TABLE 6.9 Comparison of polystyrene and polyvinyl assay plates in the ELISA for BHV1 antigen detection

Coating antibody: (µg/ml):		8		4		2	
Sample		+	−	+	−	+	−
Plate type	Sample dilution (log_{10})						
Falcon 3912 (polyvinyl)	-1	1.53	.19	1.15	.16	.64	.14
	-2	.35	.20	.33	.21	.27	.21
	-3	.17	.16	.16	.15	.17	.15
	-4	.22	.15	.17	.17	.16	.17
Dynatech M129A (polystyrene)	-1	.88	.16	.89	.17	.61	.15
	-2	.21	.18	.24	.17	.22	.17
	-3	.14	.14	.15	.15	.14	.14
	-4	.15	.15	.15	.15	.13	.14

NOTES: 1. Tabulated values are OD units

2. Test samples were infected/non infected cell culture harvests.

STATISTICAL ANALYSIS

Wilcoxon signed rank sum test for the differences (Falcon plate - Dynatech plate) at each location:

BHV1 positive sample: n = 12; t, T = 0, 78; P <0.001
BHV1 negative sample: n = 10; t, T = 4, 51; P <0.05

TABLE 6.10 Effect of plate type and storage conditions after coating, on the antigenic potency in the BHV1 IgG antibody ELISA.

Plate type:	Polystyrene						Polyvinyl					
Test sample:	Negative serum			Positive serum			Negative serum			Positive serum		
	V	C	V-C	V	C	V-C	V	C	V-C	V	C	V-C
Plate treatment after coating												
Wash and use same day	.11	.08	.03	.96	.11	.85	.22	.16	.06	1.25	.20	1.05
Wash, dry and use same day	.11	.09	.02	1.03	.12	.91	.14	.12	.02	1.09	.15	.94
Wash, dry and store 7 days 4°C	.09	.07	.02	.71	.07	.64	.11	.10	.01	.88	.11	.77
Wash, dry and store 7 days -20°C	.10	.07	.03	.74	.07	.67	.12	.10	.02	.95	.11	.84
Store wet 7 days 4°C then wash and use	.10	.08	.02	.70	.07	.63	.15	.12	.03	1.12	.13	.99
Store wet 7 days -20°C then wash and use	.09	.07	.02	.57	.08	.49	.14	.10	.04	1.12	.12	1.00

NOTES: Sera used at 1:50 dilution, conjugate 1:500
V = OD with viral antigen
C = OD with control antigen

ANOVAR FOR POSITIVE SERUM (V - C values)

Source of variation	d.f.	Sums of squares	Variance	F	P
Plate treatments	5	.1019	.0204	1.36	>0.05
Plate type	1	.1633	.1633	10.89	<0.05
Error	5	.0749	.0150		
Total	11	.3401			

TABLE 6.11. Effect of Arklone and n-butanol treatments on BHV1 ELISA antigen, and effect of gelatin post-coating of microtitre plates.

Plate Treatment with gelatin after coating	Antigen treatment	Serum: SA37 V-C	SA37 C	SA38 V-C	SA38 C	SA39 V-C	SA39 C
+	NP40	.08	.18	.56	.13	1.34	.17
+	NP40 + Arklone	.02	.21	.18	.15	.49	.24
-	NP40	.07	.16	.48	.16	1.41	.18
-	NP40 + Arklone	.03	.22	.14	.17	.43	.24
-	NP40 + Arklone + n-butanol	0		.04		.12	

NOTES: Sera SA37 = BHV1 seronegative
SA38 = BHV1 seropositive, low titre
SA39 = BHV1 seropositive
C = OD with control antigen
V-C = Calculated OD value (virus antigen - control antigen

STATISTICAL ANALYSIS (paired t-tests)

	n	t	p
Gelatin vs. no gelatin (all sera, V-C values)	6	0.83	>0.10
NP40 vs. NP40 + Arklone (SA38 & SA39, V-C values)	4	3.92	<0.05
NP40 vs. NP40 + Arklone (all sera, C values)	6	4.11	<0.01

n-butanol treated antigen lacked potency and was omitted from the analysis.

TABLE 6.12. ELISA for BHV1 antibody: identical dilutions in 96 wells on an 8 x 12 microtitre plate. Standardized row and column effects for two replicate plates.

Plate 1				Plate 2			
Rows		Columns		Rows		Columns	
A	1.9	1	-6.8	A	3.2	1	-0.5
B	2.5	2	-3.3	B	0.9	2	-0.6
C	0.3	3	2.7	C	0.1	3	1.8
D	-2.2	4	2.7	D	-1.5	4	2.0
E	-3.0	5	2.0	E	-1.3	5	2.1
F	0.2	6	2.4	G	-2.0	6	0.9
G	0.5	7	2.3	G	0.3	7	-0.4
H	-0.2*	8	0	H	0.2	8	2.0
		9	1.0			9	1.2
		10	1.1			10	0.9
		11	1.3			11	-2.6
		12	-5.5			12	-6.8
	P<0.01		P<0.001		P<0.05		P<0.001

*Replicate 1 Row H, Column 1 was an estimated value since no colour developed in this well.

TABLE 6.13. ELISA for BHV1 antibody: regression of corrected mean optical density reading on $\log_{10}$ dilution for six pairs of sera.

Plate	Serum (a)	VN Titre	Reciprocal dilution range (b)	Gradient c) ± SE	Intercept (OD)	Response at 1/100 dilution: Estimated OD	C/A	C-A	Estimated end-point reciprocal dilution in ELISA (d)
1	1A	<1	50-3200	0.125±0.023	0.42	0.17	4.8	0.64	363
	1C	8	50-3200	0.466±0.024	1.74	0.81			3306(f)
	2A	<1	50-3200	0.089±0.016	0.29	0.11	8.6	0.84	136
2	2C	24	50-6400	0.524±0.033	2.00	0.95			4226
	3A	24	50-3200	0.454±0.025	1.66	0.75	1.1	0.06	2730
	3C	8	50-6400	0.478±0.040	1.77	0.81			3117
3	4A	<2	50-6400(e)	0.170±0.009	0.63	0.29	2.6	0.46	1331
	4C	16	50-6400	0.380±0.026	1.51	0.75			5135
	5A	<2	50-400	0.060±0.018	0.15	0.03	18.3	0.52	7(f)
4	5C	24	50-6400	0.328±0.029	1.21	0.55			2422
	6A	<1	50-3200	0.095±0.013	0.34	0.15	7.9	1.04	336
	6C	>128	200-6400	0.626±0.012	2.44	1.19			5470

Notes (a) Serum A = acute, C = convalescent.

(b) Reciprocal dilution range over which log dose-response curve was linear in the ELISA.

(c) Regression coefficient and its standard error. Equation has the form:

$$OD = \text{Intercept} - \left(\text{Gradient} \times \log_{10} \frac{1}{\text{dilution}}\right).$$

(d) Dilution at which OD = 0.10, assuming linearity holds.

(e) Serum 4A: OD values at 1/200 and 1/1600 omitted due to significant deviations from linearity.

(f) Outside the linear part of the respective curves.

TABLE 6.14 ELISA for BHV1 antibody: mean optical density (OD) readings of six pairs of sera tested at a dilution of 1/100.

Mean corrected OD values for 4 blocks of 2 replicates.

	Serum*						
Plate	1A	1C	2A	2C	3A	3C	LSD+
1	0.136	0.792	0.106	0.850	0.703	0.771	0.096
2	0.183	0.783	0.099	0.832	0.671	0.746	0.091
	Serum*						
	4A	4C	5A	5C	6A	6C	
3	0.299	0.844	0.049	0.589	0.174	0.987	0.114
4	0.350	0.956	0.040	0.721	0.134	1.090	0.152

*A = Acute, C = convalescent.
+LSD = Least difference between two means necessary to show significance at the 5% level.

TABLE 6.15 ELISA for BHV1 antibody: variability between blocks/within blocks of sera.

	Plate Number			
	1	2	3	4
F ratio (18, 24 DF)	7.80	2.28	2.03	13.43
Significance (P)	<0.001	<0.05	0.05	<0.001

Notes: Plates 1 and 2 were replicates, as were plates 3 and 4. Sera were each allocated to 4 blocks at random per plate. (plates 1/2: sera 1A, 1C, 2A, 2C, 3A, 3C
plates 3/4: sera 4A, 4C, 5A, 5C, 6A, 6C).

TABLE 6.16 ELISA for BHV1 antibody: Mean optical density (OD) readings of 24 separate dilutions per plate for each of three sera

	Serum SA37 Negative		Serum SA38 Weak positive		Serum SA39 Medium positive	
Plate Number:	1	2	1	2	1	2
Day of (1	0.027	0.050***	0.254	0.216***	0.685	0.698
test (2	0.027	0.020	0.292	0.262**	0.672	0.596**
(3	0.010	0.013	0.275	0.333***	0.762	0.746
(4	0.016	0.012	0.238	0.247	0.739	0.723
SEM of 24 values	0.0032		0.0066		0.0112	
Overall mean OD	0.022		0.265		0.703	
LSD+(5% level)	0.043		0.090		0.152	
LSD+(1% level)	0.057		0.119		0.200	
95% CL++ of a single result	±0.031		±0.064		±0.108	

Significant difference between plates, ** = P<0.01, *** = P<0.001.
+LSD = least difference necessary for significance between individual dilutions within a plate.
++CL = Confidence limit.

TABLE 6.17 Frequency distribution of corrected optical density (OD) readings of sera assessed as positive or negative according to the virus neutralization test.

300 weak positive sera			652 Known negative sera		88 VN positive sera	
OD	VN < 4 n	VN ⩾ 4 n	OD	n	OD	n
0	31	6	0.00	157		
0.01-0.04	35	9	0.01	68	0.10-0.19	5
0.05-0.09	24	7	0.02	75	0.20-0.29	2
0.10-0.14	10	26	0.03	74	0.30-0.39	0
0.15-0.19	3	33	0.04	64	0.40-0.49	2
0.20-0.24	2	21	0.05	67	0.50-0.59	1
0.25-0.29	0	17	0.06	58	0.60-0.69	2
0.30-0.39	2	33	0.07	53	0.70-0.79	4
0.40-0.49		18	0.08	35	0.80-0.89	1
0.50-0.59		11	0.09	1	0.90-0.99	2
0.60-0.69		4			1.00-1.09	3
0.70-0.79		1			1.10-1.19	9
0.80-0.89		3			1.20-1.29	7
0.90-0.99		1			1.30-1.39	15
1.00-1.09		0			1.40-1.49	14
1.10-1.19		1			1.50-1.59	12
1.20-1.29		0			1.60-1.69	7
1.30-1.39		2			1.70.1.79	2
Total	107	193		652		88

TABLE 6.18 Optical density readings of purified immunoglobulin fractions of BHV1 positive bovine sera as measured in ELISA

		Serum Fractions		
		IgG1	IgG2	IgM
Assay:				
	IgG1	1.35	0.08	0.09
	IgG2	0.12	0.87	<0.01
	IgM	0.07	0.05	0.59
Protein A	Conjugate control	<0.01	0.17	<0.01

TABLE 6.19 Classification of paired serum samples from outbreaks of natural respiratory disease in cattle, according to IgM and VN antibody assays for BHV1.

The values given are the number of serum pairs in each category.

	VN negative	VN rising titre	VN positive (static or falling titre)
IgM negative (<0.25)	18	23	18
IgM rising	-	16	-
IgM static positive	-	6	6
IgM falling	-	8	13
	18	53	37

Chapter 7

GENERAL DISCUSSION

INTRODUCTION

The theme of the dissertation has been the changing pattern of disease associated with bovid herpesvirus 1 in Great Britain, with particular focus on the decade 1977-1986. From an evaluation of field data in Chapter 3, it has proceeded to detailed studies of viral isolates *in vitro* (Chapter 4) and *in vivo* (Chapter 5). Finally, the availability of experimentally infected animals for these studies has enabled a thorough evaluation of existing and new diagnostic tests (Chapter 6).

EVIDENCE FROM THE FIELD

There is a danger when a "new disease" is first reported, as happened with severe IBR in 1977-1978, that the phenomenon becomes an artificially self-sustaining epidemic fueled by positive feedback from anecdotal clinical reports (Thrusfield, 1986b). It was important therefore to seek unbiased sources of data to substantiate the claim that a virulent strain of virus had appeared, and that alternative explanations, such as the effect of husbandry changes on host-virus equilibria, were considered. Unfortunately, such unbiased data were not readily available as has been shown in Chapter 3.

The first questions to be addressed were, "Did the disease incidence suddenly increase in 1977-1978?" and "Was the disease at that time more virulent than had been seen thitherto?". Very definitely affirmative responses to both questions have been expressed to the author on many occasions at scientific and clinical meetings as well as in the VI reports and correspondence columns of the Veterinary Record

(Cuthbertson and Wood, 1979; Imray, 1979; Wiseman and others, 1979). Such views although lacking support from quantitative data should not be dismissed lightly, arising as they did from many astute and experienced practitioners and veterinary investigation officers who were well versed in the art of clinical and pathological observation.

The true population incidence of this or any other disease is almost impossible to determine. Many cases are not referred to a veterinarian and of those that are only a proportion are referred on to the VI Service. It is only at this point that a comprehensive record begins to be maintained. The cases so referred are likely to include a disproportionately high percentage of severe incidents. The VIDA II system provides a huge database of farm animal disease information for Great Britain but because it is elective, by submission from the field, rather than randomly selective as an unbiased population sample would be, it cannot offer information on absolute incidence (Thrusfield, 1986c). Providing certain precautions are taken, it can give useful information on changes in relative incidence as has been shown in Chapter 3. In particular, as advised in the preface to the annual VIDA II reports (MAFF and others, 1987), specific disease data should be related to total submissions for that species. This should even out the effects on submissions, of changes in general farming prosperity, the value of cattle and the charging policies of diagnostic laboratories. It does not compensate for an increase in specific submissions when a disease is at the forefront of attention in the veterinary and farming press. VIDA II is also weak for the recording of new diseases (Hall and others, 1980), and in the case of IBR this did not become a specifically coded category until 1980. It therefore captured only the tail end of the rising phase of the

putative epidemic curve. IBR incidence appeared to be entering a declining phase in the mid 1980s (Fig 3.1). This would be expected as the population immunity rose (Fig 3.3, Table 3.5) in response to a higher incidence of infection (Thrusfield, 1986a). A similar trend for BHV1 on a herd basis was reported by Van Nieuwstadt and Verhoeff (1983).

Samples submitted to the CVL for diagnostic virology offer a yet more biased set of data. They arise from disease outbreaks where viral aetiology is suspected in any case, and there are generally no controls for comparison with the cases. An attempt was made at statistical evaluation by relating BHV1 diagnoses to the total bovine sample submission rates (Table 3.3, Fig 3.2). The data have the particular merit of extending back to the early 1970s. There was a clear, and statistically significant, rise in the proportional diagnosis rate for IBR from 1975 to 1978. The trend was even more marked when expressed as the absolute number of diagnoses made at the CVL (Table 3.3). Other factors which could have influenced these results were changes in laboratory methods, such as from tube VN tests to microtitre systems, then subsequently to ELISA. A useful control in this case is provided by paired sera from reproductive disease cases (mainly abortion) which did not show any significant rise (Fig 3.2) in BHV1 diagnoses. This in turn supports the view that whilst BHV1 abortions do occur they have not been a prominent feature of the wave of severe IBR from 1976 onwards.

The absolute and proportional diagnosis rates by BHV1 isolation declined at the end of the study period (Table 3.3) and continued to fall in 1987-88. Although there may be an underlying population trend this was considered to be principally due to the development

work on antigen detection tests described in Chapter 6. Most VI Centres now diagnose IBR by immunofluorescence on smears or frozen sections without recourse to virus isolation or to the CVL. At this stage therefore the VIDA II data (Table 3.2), which record all VI Centre diagnoses, are more informative.

The hypothesis that the host-virus relationship for BHV1 was not in equilibrium in the study period was further supported by the rising seroprevalence both in diagnostic submissions (Table 3.5) and in export and AI tests (Table 3.4, Fig. 3.3). These indicate that between 13 and 31% of cattle were seropositive in 1986, compared with the earlier figures of 11-12% (Msolla and others, 1981; Peters and Perry, 1983) and less than 10% (Dawson and Darbyshire, 1964; Kirby and others, 1978).

Although there was a significant variation in IBR incidence from 1980-86 between counties (Fig. 3.4) it was not possible to identify the reasons for this. It was concluded that a much more detailed study would be required relating disease incidence and severity to herd types, sizes, stocking densities, husbandry practices and cattle movements.

The case records file showed that IBR affected cattle of all age groups (Table 3.7). The rising incidence in calves at the end of the study period corresponds with reports from Holland (Van Nieuwstadt and Verhoeff, 1983) which suggested an increasing role for BHV1 in the less well defined calf pneumonia complex. This may in part be accounted for by the rising seroprevalence (and the concommitant rise in prevalence of latently infected carriers) in the adult population, so that calves are increasingly likely to be exposed to BHV1

infection in their first 6 months of life. Both VIDA II and the case records analysis showed that IBR had a pronounced, and statistically significant, winter peak of incidence (Fig 3.1). The only exception to this trend was in adult cattle, in which a secondary peak in August reflected the high proportion of animals in mid gestation at that time of year. This was reflected in the August peak of BHV1-associated abortions (Table 3.10) which was the only clinical sign which differed statistically from the seasonal trend.

Five clinical signs showed trends over the 10 year period which differed significantly from the overall pattern (Table 3.9). The rise in abortions may indicate the rising prevalence of BHV1 infection in adult cattle, while the rise in pulmonary disease may reflect the consequent exposure of younger calves as discussed above. In contrast one factor leading to a decrease in the more severe signs of IBR (mortality and tracheitis) may be the control by vaccination of frank clinical disease in the herds most at risk, while the generally rising trend in population immunity may also have contributed. The fall in ocular signs may have been affected by similar factors although, like much of the above discussion, these hypotheses are speculative attempts to explain observed trends.

VIRUS CHARACTERISTICS

When the epidemic of severe IBR began in the late 1970s investigators were limited by the lack of sufficiently precise techniques with which to compare different isolates of the virus. Nevertheless, workers at Glasgow University were able to show that severe IBR was reproducible experimentally (Msolla and others, 1983c) which suggested that virulence was an attribute of the virus rather than an effect of

husbandry conditions. The latter no doubt contributed to the worst effects of the disease seen in intensive beef units in north-east Scotland (Wiseman and others, 1978).

It was first shown for human herpesviruses (Hayward and others, 1975) that RE fingerprints could reveal both major and minor differences between strains, and that the technique could be used for epidemiological studies (Buchman and others, 1979). This concept was extended to veterinary virology, for example with equine herpesvirus 1 (Allen and others, 1983), SHV1 (Paul and others, 1982) and BHV1 (Engels and others, 1981).

Misra and others (1983) first reported RE fingerprints from British strains of BHV1 and classified them as DNA types I_1 and III_1, which correspond to types 1 and 2b of Metzler and others (1985) as used in the present work. According to Misra and others (1983) the enzyme Hpa I did not distinguish between these two genotypes of BHV1. This is surprising because the present studies, supported by Mayfield and others (1983) and Kennedy and others (1986), indicate that this enzyme gives one of the clearest and most consistent distinctions between the two viral types (Fig 4.6). This clarity is facilitated by the small number of cleavage sites for the enzyme and the fact that none of them are in the short region of the genome so that the digestion fragments are all equimolar (Fig 1.2).

Eco RI and Hind III have been more widely used, and Hind III in particular distinguishes type 1 from type 2 viruses by the bands in the 7-11kbp range (Fig 4.5). Allowing for some discrepancies in molecular weight calibration, the patterns found with UK strains (Figs 4.4-4.7) agree closely with the genome maps of American strains of BHV1

(Mayfield and others, 1983). Neither the type 2a nor the encephalitic strain (type 3) patterns (Metzler and others, 1985; Brake and Studdert, 1985; Metzler and others, 1986; Engels and others, 1986) were found among UK field isolates. This conflicts with the assertion of Metzler and others (1985) that IBR and abortion isolates are of types 1 and 2a, and IPV and IPB of types 2a and 2b. In Britain type 2b was more frequently isolated from respiratory than from genital disease (Tables 4.2 and 4.3). In Northern Ireland (McFerran, personal communication; Table 4.4) and Australia (Brake and Studdert, 1985) type 2b strains predominate and are frequently found in association with respiratory disease. Both of the vaccine strains in use in Great Britain appear to be variants of type 1 virus. Although Nasalgen somewhat resembled type 2a when digested with Hind III, its Hpa I fingerprint placed it in type 1. This has been confirmed by Dr Engels (personal communication) who has studied our results (Figs. 4.10-4.12). She indicated that type 2a was more common than 2b in Switzerland and that both gave type 2 patterns with Hpa I. The vaccine strains could thus be uniquely identified by their digestion fragments with Hind III and Hpa I. This should facilitate epidemiological studies and diagnostic investigations in herds where vaccines have been used.

Workers at the Moredun Institute first obtained evidence for a change in the predominant genotype in Scotland coinciding with the emergence of more severe disease in the field (Nettleton, 1986). This was confirmed by the present studies, covering English and Welsh isolates. Although few isolates from the 1960s and early 1970s were available, neither we nor the Moredun team have found any evidence of type 1 strains in Britain before the mid 1970s. The emergence of severe

IBR has also been reported from other European countries (reviewed in Chapter 1) although an association with type 1 virus has not been documented. Metzler and others (1985) commented on the remarkable homogeneity within type 1 isolates by monoclonal antibody typing, and the present RE studies (Chapter 4) suggest that there is less genetic variability among type 1 than type 2. A plausible explanation (though lacking incontravertible proof) is that a variant or mutant of BHV1 emerged in the early 1950s in the USA (McKercher, 1959). This was the type 1 genotype which had an enhanced affinity and virulence for the respiratory tract, grew to higher titre (Gillespie and others, 1959) and was therefore able to spread rapidly through the intensive feedlots. At some stage, and probably on more than one occasion, this new virus type returned to Europe by means of latently infected cattle purchased for genetic improvement programmes. It spread rapidly, expressing its enhanced virulence whenever the epidemiological circumstances permitted. In the case of Great Britain this was in the mid 1970s, and may have been a consequence of the considerable number of imported cattle brought in for restocking following the foot and mouth disease epidemic of 1968-69. The possible association of higher prevalence of BHV1 with the presence in herds of imported Holstein cattle (Msolla and others, 1981) supports this hypothesis. Whether or not this historical view is correct, the present studies indicate the need for continued vigilance on new importations of cattle in that Britain appears to be free of both the wild-type 2a and type 3 strains of the virus.

It would be expected that the genetic differences demonstrable by RE fingerprinting would be reflected in the viral proteins. Studies at the CVL (White, 1985) with representative UK strains of DNA types

1 and 2b (strains ED1 and ED2, Table 4.1) showed small but consistent differences in the PAGE mobility of a number of viral proteins, as also reported by Pastoret and others (1980b), Misra and others (1983) and Gregersen and others (1985). Neither Gregersen and others (1985) using immunoprecipitation nor White (1985) using immunoblotting could detect antigenic differences between BHV1 types with rabbit antisera. White (1985) also showed extensive cross reactions with convalescent calf sera from experiment 2 of the present studies, reacted with electroblotted proteins from both virus types. Furthermore the cross reactivity extended to clinical protection (Table 5.8). It appears that only with the narrow epitope specificity of monoclonal antibodies will it be possible to demonstrate distinguishing antigenic features between BHV1 genotypes (Metzler and others, 1985). The application of such distinguishing monoclonals will provide a tool for the typing of isolates which is simpler than the rather specialised and elaborate RE method.

EXPERIMENTAL PATHOGENICITY

Ideally the relationship between viral genotype and virulence would be explored by comparing a large number of isolates, representing the different DNA types, in similar host groups under identical conditions. Practical considerations, not least the cost of experimental work in cattle and the time involved in clinical assessments, impose severe constraints on this approach. Although small animal models of IBR have been described (Lupton and others, 1980) they could not be used as indicators of strain virulence for cattle without first doing comparative studies in cattle and the model species. Possible confounding factors of the calf experiments include individual animal variation in an outbred population, and the difficulty with purchased

calves of obtaining animals of a uniform genetic makeup. There was evidence in experiment 2 of an age-related susceptibility to BHV1 (Fig. 5.4) which supported the findings of Msolla and others (1983a). Although not conclusive the results of experiments 3 and 5 suggested a possible host breed variation in susceptibility, which could be investigated by a breed comparison in age matched groups of calves using a standard viral inoculum. Studies in cattle would be unlikely to achieve the precision of Shanley (1984) who showed with inbred mouse strains that susceptibility to pulmonary infection with murine cytomegalovirus was inherited as an autosomal dominant associated with the H-2 locus. Further problems in the present studies may have arisen because of the practical necessity of extending the primary comparisons (experiment 1) over a period of time, with the possible effects of weather or other seasonal factors on the clinical expression of the experimental disease. This was controlled by the use of a randomized block design, although the results showed that there was not in fact any significant variation attributable to blocks (Table 5.5).

It was essential, in order to make statistical comparisons between viral strains, that a quantitative system of clinical assessment was developed. The recording system described for calf respiratory disease by Thomas and others (1977) was used, but adapted to the clinical signs expected in IBR. Essential elements were that the clinician was unaware of treatment allocations, that the same clinician did all the examinations, and that each calf was specifically examined and scored daily for each item on the check list of clinical signs. Strict attention to personal disinfection between pens was essential to prevent cross-infection and the lack of transmission to

the control calves (experiment 1) confirmed that this was effective. No significance can be attached to the absolute values of clinical score produced, as they were dependent on the clinician's personal criteria of severity for each sign, and on the weighting system used in the calculation. Nevertheless they should provide a reliable measure of relative virulence within the parameters of the experiments. Although the endeavour was always to maintain objectivity of clinical scoring one possible source of bias, notably in experiment 2, was that it was obvious to an experienced clinician that one group of calves was more severely affected than the other, and it was guessed (later proved correct) what the treatment allocations were. Thomas and others (1977) emphasised the importance of a period of pre-inoculation monitoring of experimental calves in order to determine an individual baseline for the clinical scores. This procedure was followed in the present studies but their use of individual means and tolerance limits for rectal temperature was found to be unnecessarily elaborate. The practical alternative of scoring temperature above 39°C gave a satisfactory measure of the febrile response. Thomas and others (1977) used a combination of mean scores for clinical signs and cumulative scores for rectal temperature and haematological values. The latter two were also divided by their standard deviations. The rationale for these calculations is not clear and is not explained in their paper. For the present studies the scores were originally calculated from the raw data by Thomas and others' method but this was subsequently changed to a uniformly cumulative method. This was considered more representative of the total virulence of the strain and, unlike values based on means, was independent of the length of the monitoring period provided monitoring continued until clinical recovery occurred. Thomas and others also included

values derived from weekly haematological measurements. This would not be very informative for an acute disease of short duration. As the preferred alternative of daily haematology was beyond the resources available it was omitted altogether.

There was no reason to suppose that the clinical score values for individual calves with any one virus inoculum would be anything but normally distributed. The use of analysis of variance and Student's 't' test was therefore considered appropriate as they are generally more powerful than non-parametric tests for normal data (Snedecor and Cochran, 1967). Non-parametric tests were also done (Tables 5.5-5.9) to support and confirm the validity of the conclusions of the parametric analyses. This was simpler than attempting the tedious procedures involved in testing for normality in data. A particular problem arose with experiment 1 in the need to use an estimated value to produce a balanced table for the anovar. The necessary correction factor reduced the treatment variance thereby lowering the F value and raising the significance above 5%. Friedmann's test did not require use of the estimated value and showed that there was in fact evidence of significant variation attributable to treatments (virus strains). Taken together, the clinical score results from experiments 1, 2 and 3 indicate that not only is there a variation in virulence between strains of BHV1 but that, at least within this limited series of experiments, it could be related to the genomic type as determined by RE fingerprinting. Type 1 strains produced more severe clinical signs of IBR, which observation fits the hypothesis propounded above that type 1 virus was imported to Britain in the 1970s and led to an epidemic of severe disease in the field.

For statistical analysis of the virus titres in extracted swab samples

from the experimental calves (Table 5.11-5.18) non-parametric tests were used in order to avoid distortion due to the large number of negative samples, whose titre could properly only be described as "<1" where there was no detectable growth from the undiluted sample. As with the clinical scores cumulative virus scores derived from the logarithms of the titres were used to facilitate comparisons between strains. The virus score was affected by both the height and duration of the virus shedding curve (Fig 5.6) but as both of these are relevant to the risk of cross infection between animals it was considered a pertinent measure.

In experiment 1 there was a significant variation in virus score attributable to strains and this appeared to be related to viral genotype when the strains were grouped according to their RE profiles (Table 5.11). The analysis was further supported by a detailed analysis of individual titres using the Kruskal Wallis method (Table 5.12) which showed significant variation attributable to strains, genotype group, and (not surprisingly) days post-inoculation. The same pattern of virus excretion was repeated in the age-matched pair comparison (Table 5.14) in which calves inoculated with DNA type 1 virus had significantly higher cusum titres in the nasal mucus as shown both by the paired Wilcoxon test and by a Mann Whitney comparison of the medians. The differences were significant individually for only two of the pairs, while an overall comparison of daily titres was not significant. These analyses emphasise the importance of careful selection of statistical methods which will highlight the differences between treatments and distinguish them from the confounding effects of other variables. In experiment 3 the differences in virus shedding between the two strains of DNA type 1 were not significant, but in

view of the very limited number of observations this can only be regarded as a provisional conclusion (Table 5.18).

The measurement of viral titres in the swab extraction media gave a reasonable estimate of the infectious titre per gram of nasal mucus, in view of the consistent amount of mucus collected on the swabs (Table 5.10). Virus shedding into the environment would also be affected by the total mucus production which tended to be higher with type 1 strains (Table 5.7). Less consistent sampling was achieved by ocular swabbing (Table 5.10) and a larger sample was likely to be obtained from eyes with frank discharge than from clinically normal ones. Accordingly, although some differences in virus titres were shown (Tables 5.13, 5.15), the results were ascribed less importance than those from the nasal swabs.

It is evident from Table 5.16 that recrudescent infections of the genital tract can follow a primary respiratory infection. This is of importance when selecting bulls for breeding or AI purposes and supports the view that any serological reactor is a potential risk for spread of virus in semen. In the context of control of BHV1 another important finding is that recovered cattle, whilst immunologically protected against clinical disease (Table 5.8), can still become infected with BHV1 of another genotype, and be an infective risk for other cattle (Table 5.17). The pattern of excretion was similar to that shown by vaccinated calves (Frerichs and others, 1982).

One of the major features of IBR in the field in Britain in the study period was the apparent increase in incidence. These virological studies offer an explanation for the phenomenon in that animals in-

fected with the "new" strain (DNA type 1) excrete much higher titres of virus (1 to 2 logs at the peak in some cases - Table 5.14) and are therefore more likely to transmit infection than are animals infected with type 2 strains.

This provides a parallel with earlier comparisons of IPV and IBR strains (Gillespie and others, 1959) where a 100-fold difference in titre was noted. It is probable (as indicated in Table 4.4) that they were also studying viruses of types 1 (IBR) and 2 (IPV).

ANTIBODY RESPONSES

The development of the ELISA (described in chapter 6), initially for measurement of IgG responses and subsequently for immunoglobulin class and subclass specific assays, enabled a more detailed study of the serological responses to experimental infection than had previously been possible. The calves showed a classic sequence of antibody responses with an early rise in IgM (day 8) followed within a few days by IgG1 and IgG2 (from days 9-10) which rose to a peak or a plateau as the IgM fell from its peak at day 14. IgA responses were detected only weakly in nasal secretions and not at all in serum.

Although IgG1 is present in considerable amounts in bovine respiratory tract secretions, IgA is the dominant immunoglobulin especially in the upper respiratory tract (Butler, 1983). A strong secretory IgA response would therefore be expected following an upper respiratory infection such as BHV1 (Gerber and others, 1978). The lack of known positive standards for the IgA assay led to difficulties in calibration of the test. It is possible that the commercial antiserum used was insufficiently potent for the purpose, although the antisera for IgM, IgG1 and IgG2 from the same manufacturer appeared to have ade-

quate specificity and potency, as shown in Chapter 6. Further development and characterisation of the IgA assay is needed before it can be used to study this aspect of the immune response.

DIAGNOSIS

Because IBR is principally a disease of the upper respiratory tract, samples for diagnosis are readily collected and the confirmation of BHV1 is generally easily achieved. As shown in Chapters 5 and 6 the virus is present in high titres in the nasal secretion during the first week after infection. Although elaborate sampling techniques have been described for bovine respiratory disease diagnosis (Thomas and Stott, 1975; Kimman and others, 1986), and may be necessary for pneumonic infections, a simple nasal swab technique as described in Chapter 6 was quite adequate in the case of IBR. The addition of a liquid transport medium facilitated transport to the laboratory and improved the quality of cytological smears for direct immunofluorescence compared with unwashed nasal mucus smears, even though the latter have been very successfully employed (Nettleton and others, 1983). It is clear from the experimental infections that nasal secretion samples for antigen detection must be taken early in the course of the disease - ideally within a week of first exposure to the virus, which probably corresponds in field cases to a 4-5 day window from the first detection of clinical signs. The use of a rectal thermometer to detect in-contact animals in the prodromal febrile phase would be of benefit in the investigation of outbreaks in groups of cattle.

The choice of antigen detection system appears to matter little provided that the test conditions are optimised. Direct immunofluorescence was selected for routine IBR diagnosis in the MAFF laboratory

service on the grounds that it was technically simple and familiar to the staff. For the labelling of cytological smears immunoperoxidase could be used but its advantages (visible light microscopy, permanent preparations) were outweighed by the drawbacks (high noise levels, endogenous enzyme problems, a longer test with more steps). The immunoperoxidase method has a particular advantage for frozen or paraffin embedded tissue sections where it can be used to relate the presence of viral antigen to tissue pathology (Higgins and Edwards, 1986).

For other laboratories, methods aimed at the detection of soluble viral antigen may be preferred. ELISA could be amplified to a high sensitivity by using the biotin-avidin system, but even so it only equalled the technically much simpler RPHA system. An advantage of these methods is the facility with which large batches of samples can be processed. This may be of little benefit to many laboratories. The methods also avoid the need, inherent in cytological techniques, for skilled staff to read and interpret the test, although this could be considered a drawback which might lead to a higher risk of false positives when relying on a purely mechanical determination of positivity.

Future developments in antigen detection systems (whatever the label) are likely to involve virus specific monoclonal antibodies (Collins and others, 1985a). The high specificity of these reagents leads to low noise levels in the assays and hence to high sensitivity. Nevertheless the low noise and high signals obtained with polyclonal gnotobiotic calf sera in the present studies will prove hard to beat. Monoclonals also have a potential for use in virus sub-typing where this is thought desirable (Metzler and others, 1985).

DNA hybridization has been heralded as the answer to highly sensitive detection of the virus (Petterson and Hyypia, 1985). At present the technique is more lengthy and elaborate than antigen detection, while the reliance on radio labels (Pacciarini and others, 1986) for all the most sensitive protocols rules it out for practical diagnostic applications. Furthermore, problems may arise in clinical samples from the high proportion of non-viral material present leading to reduced sensitivity of virus detection with DNA probes (Dorman and others, 1985).

Serology continues to provide a useful tool for the diagnosis of BHV1. The VN test is simple to perform, provides a high degree of specificity and is likely to remain the standard against which other assays are evaluated (Office International des Epizooties, 1986). The ELISA is an economical alternative which permits more rapid screening of sera for regulatory and diagnostic purposes. Its sensitivity approaches that of the enhanced VN test using a 24 hour neutralization time (Bitsch, 1978; Edwards and others, 1986b). It is difficult to purify herpesvirus antigens so as to be completely free of host cell proteins (Spear and Roizman, 1972). It was shown in this study that satisfactory and specific results were obtainable using a crude detergent-extracted antigen from BHV1-infected cell cultures. The incorporation of a control cell antigen as part of the test provides an internal baseline for each individual serum. The value of this was shown by the considerable variation among field sera in the background OD obtained with the control antigen.

The results of serological ELISAs can be expressed in a variety of ways, none of which is completely satisfactory (de Savigny and Voller, 1980). Both end-point titrations and OD measurements at a single

dilution are influenced in a complex manner by the concentration and the affinity of the antibodies present in the test sample (Butler and others, 1978; Lehtonen and Eerola, 1982; Lew, 1984). For diagnostic purposes, the mathematical form of the result is of little importance provided some sort of quantitative expression is available which can be evaluated against established parameters. The OD result at a fixed dilution provides such a measure, and is easily calculated. The extensive calibration of the test described in Chapter 6 provided the necessary parameters.

Diagnostic serology should be considered from two distinct points of view. In the first instance the test is used for regulatory purposes (pre-export tests, AI health controls, other herd health control schemes and surveillance work). For such applications only a qualitative result is required, but the test sensitivity should be maximised and the positive/negative cut-off point determined with the greatest possible precision. This was achieved by testing sera at a dilution of 1/50, and by calibrating the assay with a large population of known negative samples. It is important that those using such test results appreciate that a fixed cut-off value such as 0.10 OD units, as used here, is arbitrary and inevitably a few sera will be misclassified by such a system. Although in population terms this may not matter it could cause problems particularly when dealing with individually valuable animals.

The second main application for serology is in disease diagnosis. A single sample from an affected case is uninformative as positive results indicate past infection but not how recent it was, while negative results can mean either no exposure, or that an acutely affected animal has not yet seroconverted. Paired acute and convales-

ent sera are therefore essential for diagnosis, a concept that is well known. Conventionally, a fourfold rise in titre is considered the minimum criterion for diagnosis of active infection. For the ELISA using OD values as the quantitative result it was necessary to establish equivalent parameters for a diagnostic rise, for which purpose the "least significant difference" method was used. The chosen criterion of a 0.20 rise in OD value was shown to compare well with a fourfold rise in VN titre.

Further development of serological diagnosis should focus on the detection of viral-specific IgM class antibody. It was established in Chapter 5 that IgM is detectable 8 days after infection, peaks at 12-14 days then falls to low or undetectable levels by 3-4 weeks. The finding of high IgM would therefore provide a useful diagnostic indicator of recent active infection with the virus, and could obviate the need for collection of convalescent sera for the demonstration of rising titres. Similar findings have been reported for bovine respiratory syncytial virus (Westenbrink and Kimman, 1987) and vesicular stomatitis virus (Vernon and Webb, 1985). IgM assays are particularly prone to difficulties caused by high non-specific background reactions (Tedder, 1984), as was encountered in the study of field sera described in Chapter 6. Although the inversion of the assay to an antibody capture system (Tedder, 1984) provided some improvment, it remains difficult to find or prepare polyclonal antisera of adequate specificity against IgM. (Butler, 1983). Monoclonal antibodies are the obvious answer. They have already been applied in a respiratory syncytial virus IgM assay in cattle (Kimman and others, 1987; Westenbrink and Kimman, 1987) and the preliminary studies reported here show their potential for a sensitive and specific assay for

bovine IgM applicable to BHV1 diagnosis.

The studies reported here have shown that a variety of immunoassays may be employed in BHV1 diagnosis, using fluorescent, enzymic or erythrocyte labels and applying them to both antigen and antibody detection. The choice of method for an individual laboratory depends on the facilities available, the experience of the personnel, the nature and the number of diagnostic submissions. All the methods depend upon the availability of high quality monospecific antisera, and this is an area where monoclonal antibodies should play a prominent role in future.

PRINCIPAL CONCLUSIONS

1. Infectious bovine rhinotracheitis was first described in Great Britain in 1961 although there is no reason to suppose it was not present before that date. In the late 1970s it appeared in epidemic form with greater virulence and a stronger propensity to spread. In this respect it resembled IBR as described in the USA rather than the milder form seen previously in Britain.

2. The incidence of severe IBR appears to have declined in recent years. This may reflect the successful application of health control regimens (including vaccination) and also a rising bovine population immunity, which is reflected in the data for seroprevalence.

3. Molecular genomic studies of viral isolates indicated that severe IBR was associated with BHV1 of genotype 1 which closely resembles American IBR isolates. The evidence suggests that virus of this genotype, although now predominant, was absent from Great Britain before the mid 1970s. It may still be absent

from Northern Ireland.

4. Experimental infections in calves showed that genotype 1 viruses were more virulent than genotype 2 isolates of BHV1. Calves infected with genotype 1 also shed significantly higher titres of virus, which may explain the propensity of this virus to spread to other cattle. Calves recovered from infection with either genotype were clinically resistant to challenge with the other type.

5. The serological responses of experimentally infected calves followed a consistent pattern. ELISAs were developed to measure the immunoglobulin classes of the responses. IgM was detected from day 8 after infection, peaked at days 12 to 14, then fell to low or undetectable levels by 3 to 4 weeks after infection. IgG (sub classes 1 and 2) and neutralizing antibody responses appeared at days 9 or 10 and rose to a high level by 2 to 3 weeks. This persisted for at least several months. Recovered calves given dexamethasone to reactivate latent virus showed anamnestic rises in IgG and neutralizing antibody, as did calves challenged with virus of a heterologous genotype.

6. Diagnostic test procedures were developed using samples from the experimentally infected calves. The most promising of the tests were evaluated on field materials then brought into routine use at the laboratory. These included antigen detection procedures (immunofluorescence, enzyme immunoassays) and serology (ELISA). Other assays showed potential which might be best exploited by the application of monoclonal antibodies (reverse passive haemagglutination for antigens, detection of IgM antibody responses).

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APPENDIX 3 (i) County statistics for cattle and for IBR/IPV incidence (England)

Sources of data: Agricultural Census, 1982
VIDA II, 1980-86

County	Total cattle (1000s)	Total agricultural area (1000s of Ha.)	No. cattle holdings	Mean no. cattle per Ha.	Mean no. cattle per holding	IBR/IPV (systemic) incidents 1980-86	IBR/IPV (systemic) per 100,000 cattle	IBR/IPV (fetopathy) Incidents 1980-86	IBR/IPV (fetopathy) per 100,000 cattle
Avon	127	90	1532	1.41	83	23	18.1	3	2.4
Bedfordshire	32	93	434	0.34	74	19	59.4	1	0
Berkshire	54	75	508	0.72	106	18	33.3	1	1.9
Buckinghamshire	120	134	1260	0.90	95	25	20.8	0	0
Cambridgeshire	44	293	820	0.15	54	52	118.2	3	6.8
Cheshire	295	176	3240	1.68	91	276	93.6	26	8.8
Cleveland	25	32	322	0.78	78	29	116.0	3	12.0
Cornwall	416	286	5566	1.45	75	29	7.0	15	3.6
Cumbria	594	464	5560	1.28	107	220	37.0	8	1.3
Derby	221	186	3050	1.19	72	75	33.9	5	2.3
Devon	696	526	8694	1.32	80	248	35.6	31	4.5
Dorset	268	202	2202	1.33	122	71	26.5	5	1.9
Durham	137	153	1789	0.90	77	36	26.3	2	1.5
E. Sussex	106	119	1270	0.89	83	33	31.1	6	5.7
Essex	79	273	1043	0.29	76	46	58.2	9	11.4
Gloucestershire	190	210	2172	0.90	87	155	81.6	19	10.0
Gt. London	11	17	149	0.65	74	1	9.1	0	0
Gt. Manchester	45	43	931	1.05	48	1	2.2	0	0
Hampshire	172	230	1635	0.75	105	87	50.6	21	12.2
Hereford/Worcs.	308	321	4172	0.96	74	180	58.4	0	0
Hertforshire	49	107	567	0.46	86	39	79.6	1	2.0
Humberside	99	292	1548	0.34	64	29	29.3	2	2.0
Isle of Wight	28	27	313	1.04	89	14	50.0	0	0

contd....

APPENDIX 3 (i) Contd.

County	Total cattle (1000s)	Total Agricultural Area (1000s of Ha.)	No. cattle holdings	Mean no. cattle per Ha.	Mean no. cattle per holding	IBR/IPV (systemic) incidents 1980-86	IBR/IPV (systemic) per 100,000 cattle	IBR/IPV (fetopathy) incidents 1980-86	IBR/IPV (fetopathy) per 100,000 cattle
Kent	108	264	1563	0.41	69	43	39.8	2	1.9
Lancashire	290	228	3749	1.27	77	158	54.5	4	1.4
Leicestershire	191	200	2074	0.96	92	34	17.8	0	0
Lincolnshire	151	519	2202	0.29	69	50	33.1	0	0
Merseyside	13	20	160	0.65	81	16	123.1	0	0
Norfolk	135	424	1886	0.32	72	50	37.0	1	0.7
Northamptonshire	118	191	1271	0.62	93	33	28.0	5	4.2
Northumberland	247	383	1941	0.64	127	14	5.7	4	1.6
North Yorks	502	631	6435	0.80	78	277	55.2	36	7.2
Nottinghamshire	90	154	1159	0.58	78	31	34.4	2	2.2
Oxfordshire	138	209	1380	0.66	100	48	34.8	2	1.4
Salop	342	292	3836	1.17	89	162	47.4	1	0.3
Somerset	405	284	4232	1.43	96	165	40.7	6	1.5
S. Yorks	54	83	856	0.65	63	42	77.8	1	1.9
Staffordshire	284	204	3603	1.39	79	145	51.1	13	4.6
Suffolk	96	306	1252	0.31	77	33	34.4	6	6.3
Surrey	66	73	849	0.90	78	38	57.6	2	3.0
Tyne & Wear	13	18	170	0.72	76	3	23.1	0	0
Warwickshire	128	160	1530	0.80	84	50	39.1	0	0
West Midlands	17	19	284	0.89	60	7	41.2	0	0
West Sussex	113	128	1027	0.88	110	43	38.1	3	2.7
West Yorkshire	104	102	2003	1.02	52	62	59.6	2	1.9
Wiltshire	254	276	2177	0.92	117	151	59.4	8	3.1
Unknown						7		-	

APPENDIX 3 (ii) County/Regional statistics for cattle and for IBR/IPV incidence (Wales and Scotland)

Sources of data: Agricultural Census, 1983
VIDA II, 1980-86

County/ Region	Total Cattle (1000s)	Total Agricultural Area (1000s of Ha.)	No. Cattle Holdings	Mean No. Cattle per Ha.	Mean No. Cattle per Holding	IBR/IPV (systemic) incidents 1980-86	IBR/IPV (systemic) per 100,000 cattle	IBR/IPV (fetopathy) incidents 1980-86	IBR/IPV (fetopathy) per 100,000 cattle
Powys	270	455	3916	0.59	69	30	11.1	3	1.1
Gwynedd	187	320	2860	0.58	65	22	11.8	3	1.6
Dyfed	550	480	7855	1.15	70	67	12.2	6	1.1
Clwyd	211	195	2436	1.08	87	49	23.2	10	4.7
S. Glamorgan	35	25	284	1.40	123	3	8.6	0	0
N. Glamorgan	34	59	541	0.58	63	17	50.0	0	0
W. Glamorgan	30	43	467	0.70	64	4	13.3	0	0
Gwent	104	93	1204	1.12	86	58	55.8	5	4.8
Shetland	5	131)		0.04)		5	100.0	0	0
Orkney	92	79)	6841	1.16)	40	44	47.8	2	2.2
W Isles	8	243)		0.03)		0	0	0	0
Highland	169	1996)		0.08)		83	49.1	14	8.3
Grampian	513	616	6258	0.83	82	95	18.5	11	2.1
Tayside	175	616)		0.28)		104	59.4	3	1.7
Fife	79	94)	5269	0.84)	91	36	45.6	0	0
Lothian	68	126)		0.54)		36	52.9	1	1.5
Borders	160	380)		0.42)		125	78.1	5	3.1
Central	76	190)		0.40)		11	14.5	2	2.6
Strathclyde	535	979)	11602	0.55)	91	88	16.4	9	1.7
Dumfries & Galloway	446	450)		0.99)		116	26.0	21	4.7

APPENDIX 3 (iii) Distribution of VIDA II records and CVL case records by VI Centre

VI Centre	Total IBR/IPV (VIDA 1980-86)	IBR case records (Virology Dept, CVL)	
		1977-79	1980-86
Aberystwyth	10	2	5
Bangor	90	3	22
Bristol	299	19	4
Cambridge	190	3	8
Cardiff	62	4	6
Carmarthen	73	7	49
Chester	32	3	19
Gloucester	335	9	9
Leeds	203	9	23
Lincoln	64	12	36
Liverpool	441	16	16
Newcastle	41	4	22
Northampton	65	5	58
Norwich	76	4	13
Penrith	238	7	8
Reading	92	-	7
Shrewsbury	339	40	16
Starcross	260	10	18
Sutton Bonington	134	17	60
Thirsk	283	12	93
Truro	43	-	18
Weybridge	24	4	9
Winchester	177	3	11
Worcester	229	14	133
Wye	90	3	9
Aberdeen	95	-	-
Auchincruive	89	-	-
Dumfries	115	-	1
Edinburgh	81	-	1
Inverness	85	-	-
Oban	4	-	-
Perth	150	-	2
St Boswells	120	-	-
Thurso	83	-	-
Non-VIC origin	-	-	2

APPENDIX 4(i) Geometric mean values for fragment size (Kbp) following EcoRI digests of BHV1 DNA

Virus strain:	ED1 mean[(a)]	CL[(b)]	ED2 mean	CL	ED3 mean	CL	ED4 mean	CL	ED5 mean	CL	ED6 mean	CL
	47.9	60.3 38.1	47.5	60.3 37.4	47.5	60.3 37.4	44.8	57.6 34.9	47.8	62.7 36.4	46.5	60.7 35.6
	23.1	25.1 21.1	22.9	25.1 20.9	23.0	25.5 20.7	23.0	25.6 20.6	22.9	25.7 20.3	21.9	24.8 19.4
	17.5	18.8 16.2	19.6	21.2 18.1	18.6	20.1 17.1	16.8	18.2 15.5	17.4	19.0 15.9	19.5	22.0 17.2
	16.8	17.8 15.9	18.0	19.1 16.9	16.7	17.9 15.5	14.4	15.9 13.0	16.6	18.1 15.2	13.8	14.9 12.7
	14.0	14.4 13.6	13.8	14.4 13.3	14.0	14.9 13.2	8.8	9.1 8.5	13.8	14.8 12.9	7.7	8.1 7.3
	8.8	9.1 8.6	8.9	9.2 8.6	8.8	9.1 8.5	3.2	3.8 2.8	8.7	9.0 8.3		
	3.1	3.3 3.0			3.1	3.3 3.0			3.1	3.2 3.0		

Notes (a) Each value is calculated from measurements of 4 gels

(b) 95% confidence limits

APPENDIX 4(ii) Geometric mean values for fragment size (Kbp) following Hind III digests of BHV1 DNA

Virus strain:	ED1 mean(a)	ED1 CL(b)	ED2 mean	ED2 CL	ED3 mean	ED3 CL	ED4 mean	ED4 CL	ED5 mean	ED5 CL	ED6 mean	ED6 CL
	22.2	25.0 19.6	22.2	25.2 19.6	22.1	24.8 19.6	21.4	23.7 19.3	21.7	24.0 19.6	21.8	23.6 20.1
	20.2	22.7 17.9	20.2	22.8 17.9	20.2	22.3 18.4	19.6	21.4 17.9	19.9	21.5 18.4	19.9	21.2 18.7
	16.9	18.8 15.3	17.2	19.5 15.2	17.6	19.4 15.9	16.2	17.7 14.9	16.7	18.0 15.5	16.6	18.3 15.1
	15.8	17.4 14.2	16.2	17.9 14.6	16.4	17.9 15.0	15.0	16.3 13.8	15.5	16.6 14.5	14.4	15.5 13.3
	14.4	15.7 13.1	14.0	15.5 12.7	14.5	15.8 13.3	14.0	15.0 13.1	14.4	15.4 13.5	12.7	13.3 12.0
	13.6	14.9 12.5	12.6	13.8 11.5	12.5	13.5 11.6	13.0	14.0 12.1	13.4	14.1 12.6	10.9	11.9 10.0
	12.4	13.4 11.5	10.7	11.5 9.9	9.4	10.0 8.8	12.2	13.0 11.4	12.2	12.9 11.6	9.5	10.2 9.0
	9.4	10.0 8.7	9.4	10.0 8.8	8.7	9.3 8.3	9.2	9.7 8.6	9.3	9.8 8.8	8.2	9.0 7.5
	8.7	9.3 8.2	8.0	8.5 7.6	8.1	8.5 7.6	8.6	9.0 8.1	8.6	9.0 8.3	7.6	7.7 7.5
	8.1	8.6 7.7	7.6	8.1 7.1	4.1	4.4 3.9	8.0	8.2 7.8	8.0	8.3 7.7	4.0	4.2 3.8
	4.2	4.5 4.0	4.3	4.6 4.0			4.1	4.4 3.8	4.1	4.4 3.8		
			2.85	3.0 2.7								

Notes (a) Each value is calculated from measurements of 4 gels
(b) 95% confidence limits

APPENDIX 4(iii) Geometric mean values for fragment size (Kbp) following HpaI digests of BHV1 DNA

Virus strain:	ED1 mean(a)	CL(b)	ED2 mean	CL	ED3 mean	CL	ED4 mean	CL	ED5 mean	CL	ED6 mean	CL
	44.6	48.1 41.4	44.5	47.3 41.9	45.4	47.5 43.4	46.2	48.6 43.9	47.3	49.8 44.9	47.1	50.4 43.9
	26.6	27.4 25.8	26.3	26.9 25.7	27.2	27.7 26.8	27.7	28.7 26.8	27.8	28.8 26.9	26.6	27.6 25.7
	19.8	20.0 19.5	22.9	23.4 22.4	19.9	20.3 19.6	20.1	20.7 19.6	20.0	20.9 19.0	23.0	24.0 22.1
	16.4	16.7 16.2	16.3	16.6 16.1	16.6	16.9 16.3	16.9	17.5 16.2	16.7	17.3 16.2	16.4	17.1 15.9
	10.7	11.0 10.5	3.5	3.6 3.4	10.7	10.9 10.6	10.8	10.9 10.7	10.9	11.1 10.7	3.6	3.7 3.5
	9.2	9.4 9.0			9.3	9.5 9.1	9.4	9.7 9.1	9.4	9.6 9.2		
	4.5	4.6 4.4			4.6	4.7 4.5			4.6	4.8 4.4		

Notes (a) Each value is calculated from measurements of 4 gels

(b) 95% confidence limits

APPENDIX 4(iv) Geometric mean values for fragment size (Kbp) following Bam HI digests of BHV1 DNA

Virus strain:	ED1 mean	ED1 CL(a)	ED2 mean	ED2 CL	ED3 mean	ED3 CL	ED4 mean	ED4 CL	ED5 mean	ED5 CL	ED6 mean	ED6 CL
No. of gels measured :	5		3		5		4		4		4	
	29.3	30.2 28.5	28.7	30.1 27.4	29.4	30.3 28.5	29.4	30.4 28.6	29.3	31.3 27.4	29.1	30.4 27.9
	22.0	22.5 21.6	19.1	19.6 18.6	22.5	23.0 22.0	21.8	22.0 21.6	21.7	22.6 20.9	22.2	22.9 21.6
	19.4	19.9 19.0	18.2	18.7 17.7	19.5	19.8 19.2	19.9	20.2 19.5	19.4	19.9 19.0	19.5	20.0 19.0
	18.2	18.5 17.9	16.7	17.1 16.3	18.2	18.5 17.9	18.1	18.5 17.7	18.0	18.3 17.7	16.6	16.9 16.3
	16.6	16.8 16.4	13.9	14.2 13.7	16.4	16.8 16.2	16.7	17.0 16.4	16.4	16.6 16.2	13.3	13.6 13.1
	12.8	13.1 12.6	13.5	13.8 13.2	12.8	13.2 12.4	12.7	12.8 12.6	12.6	13.0 12.3	12.8	13.1 12.5
			12.8	13.2 12.5			12.1	12.2 12.0			4.6	4.8 4.4
			8.6	8.9 8.4								
			4.6	6.1 3.5								

Notes (a) 95% confidence limits

APPENDIX 4(v) Geometric mean values for fragment size (Kbp) following PstI digests of BHV1 DNA

Virus strain:	ED1 mean	ED1 CL(a)	ED2 mean	ED2 CL	ED3 mean	ED3 CL	ED4 mean	ED4 CL	ED5 mean	ED5 CL	ED6 mean	ED6 CL
No. of gels measured :	5		5		5		4		3		4	
	8.1	8.3 8.0	8.1	8.1 8.0	8.1	8.2 8.0	8.0	8.2 7.9	8.1	8.2 7.9	8.1	8.2 8.0
	6.8	7.0 6.7	6.7	6.9 6.6	7.0	7.1 6.9	6.5	6.6 6.5	6.9	7.0 6.7	6.9	7.0 6.7
	6.6	6.7 6.4	6.5	6.7 6.4	6.6	6.6 6.5	6.0	6.2 5.8	6.6	6.7 6.4	6.5	6.7 6.4
	6.0	6.1 5.9	5.9	5.9 5.8	6.0	6.1 5.9	4.5	4.7 4.3	6.0	6.1 5.9	5.8	5.9 5.7
	4.5	4.7 4.4	4.7	4.9 4.6	4.9	5.0 4.7			5.8	5.9 5.7	4.7	4.9 4.6
									4.9	5.0 4.7	4.0	4.2 3.8

Notes (a) 95% confidence limits

APPENDIX 4(vi) Geometric mean values for fragment size (Kbp) following Bst EII digests of BHV1 DNA

Virus strain:	ED1 mean	ED1 CL(a)	ED2 mean	ED2 CL	ED3 mean	ED3 CL	ED4 mean	ED4 CL	ED5 mean	ED5 CL	ED6 mean	ED6 CL
No. of gels measured:	5		4		5		5		5		5	
	37.0	39.0 35.1	36.3	39.0 33.8	36.3	38.4 34.3	37.2	38.9 35.7	37.9	40.0 35.4	37.4	39.6 35.2
	28.0	28.8 27.2	28.0	29.5 26.6	28.6	29.6 27.6	29.5	30.4 28.6	29.1	29.7 28.4	28.3	29.1 27.5
	18.8	19.3 18.3	23.6	24.8 22.6	19.5	19.9 19.0	19.3	19.6 19.1	19.4	19.7 19.1	23.7	24.0 23.3
	13.2	13.5 12.8	22.6	23.5 21.8	10.8	10.9 10.6	10.4	10.6 10.2	10.5	10.8 10.2	22.4	23.0 21.8
	10.1	10.5 9.8	15.6	16.2 14.9	8.8	9.0 8.7	8.8	9.0 8.7	9.1	9.3 8.9	15.6	15.9 15.3
	8.9	9.0 8.7	14.0	14.4 13.7	8.5	8.6 8.3	8.5	8.6 8.4	8.5	8.7 8.3	14.1	14.3 14.0
	8.5	8.7 8.3	8.5	8.7 8.2	6.4	6.6 6.3	6.5	6.6 6.4	6.5	6.6 6.4	8.3	8.5 8.2
	6.4	6.6 6.3	6.4	6.7 6.2	4.8	5.5 4.3	4.2	4.6 3.9	4.4	5.1 3.9	6.5	6.5 6.4
	4.7	4.9 4.6	4.3	4.6 4.1	4.4	5.9 3.3					4.3	4.5 4.1
	4.2	4.4 4.0										

Notes (a) 95% confidence limits

APPENDIX 5(i)

Cumulative 11 day clinical scores for individual calves in experiment 1, by clinical signs

Viral inoculum:	ED1			ED2			ED3			ED4			ED5			ED6			Control			
Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	A	B	C
Apathy	5							5					5				5					
Inappetance		15	21					20	23		5	41		5	10		10				5	
Diarrhoea							2						2									
Dyspnoea			2					2														
Cough	6					8	16	6	2			2	6			2	2					
Adenitis							12			2			2									
Rhinitis	18	20	16		6	6	18	14	14		22	16	18	16			28	2				
Nasal discharge	5	36	22		28	12	26	22	20	8	32	12	8	30	6		17	8				
Ocular discharge:																						
(R)	4	7	3		1	2	3	4		8	5	12		3		5	3	2				
(L)	16	3	13	1	7	6	18	7	9	12	19	23	9	13	10	10	4	3				
Conjunctivitis:																						
(R)	6	6			2		7	1	1	8	3	15	8	5		8		1				
(L)	21	22	10	3	11	11	22	9	18	15	17	28	19	16	4	12	12	3				
Respiratory rate	2		8			6						14				6			4			
Rectal temperature	83	137	71	25	27	27	83	137	102	60	24	122	57	23	21	42	37	17	2			
TOTALS	166	246	166	29	82	78	207	227	189	113	127	285	134	111	51	85	118	36	6	0	5	0

APPENDIX 5(ii)

Daily total clinical scores for individual calves in experiment 1

Viral inoculum:	ED1			ED2			ED3			ED4			ED5			ED6			Controls			
Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	A	B	C
Days after inoculation:																						
1	0	8	4	0	4	2	2	6	7	0	0	4	4	6	1	7	2	4	0	0	0	0
2	18	24	23	3	5	6	16	19	10	17	6	12	13	1	8	17	27	7	0	0	0	0
3	24	32	24	3	14	15	29	34	30	21	23	30	23	23	3	17	29	9	0	0	0	0
4	22	33	42	2	17	27	29	47	32	22	17	47	28	17	13	15	27	11	0	0	0	0
5	27	29	24	4	23	11	36	39	26	21	11	31	22	23	15	12	21	10	2	2	5	1
6	25	39	21	0	16	9	31	29	30	12	21	38	19	17	5	6	12	11	3	0	0	2
7	17	36	12	2	4	6	30	19	16	8	12	33	17	10	5	4	7	5	2	0	0	0
8	19	28	5	3	5	3	32	22	29	6	13	27	10	18	6	4	12	4	2	1	0	3
9	15	34	10	8	4	9	22	19	18	6	11	24	11	11	4	1	7	3	0	1	0	2
10	4	30	5	1	2	7	11	24	16	2	8	21	5	7	3	3	6	6	2	0	0	2
11	4	31	3	3	0	4	6	9	17	0	5	27	4	3	1	6	2	4	0	1	0	0
		*										*										

Notes: The sum of the daily scores for each calf does not equal the calculated total cumulative value, as the daily scores do not take into account the pre-inoculation readings.

* Nasal discharges in these calves persisted beyond 11 days.

APPENDIX 5(iii)

Virus titre (log_{10} $TCID_{50}$/90µl extraction medium) in daily nasal swab samples (experiment 1)

Virus	Replicate	Days after inoculation											
		0	1	2	3	4	5	6	7	8	9	10	11
ED1	A	-	2.25	5.75	4.75	5.00	4.75	4.00	1.25	0.75	-	-	-
	B	-	1.75	5.00	5.00	4.25	5.25	3.00	2.50	1.75	1.75	-	-
	C	-	3.50	4.00	3.75	3.25	4.00	2.75	-	-	-	-	-
ED2	A	-	3.50	5.25	4.25	3.50	3.25	1.75	0.75	-	-	-	-
	B	-	-	3.75	4.25	-	5.25	2.00	1.50	-	-	-	0.75
	C	-	2.00	4.00	3.75	2.50	2.50	2.25	0.50	-	-	-	-
ED3	A	-	2.00	6.75	5.25	5.25	3.75	4.50	3.25	1.25	-	-	-
	B	-	2.50	4.75	3.50	3.75	5.25	4.00	1.50	1.00	0.75	-	-
	C	-	3.50	5.75	3.75	4.75	4.00	2.00	1.25	-	-	-	-
ED4	A	-	2.75	5.50	5.50	5.00	5.25	2.75	0.75	1.00	-	-	-
	B	-	2.00	2.25	2.50	3.75	5.25	4.25	1.75	1.75	0.75	-	-
	C	-	2.50	5.50	3.25	3.50	3.25	3.00	2.50	-	2.00	-	-
ED5	A	-	-	5.50	4.75	5.75	5.00	4.00	2.75	0.75	1.00	0.75	-
	B	-	1.50	5.00	3.25	4.00	4.50	4.00	1.75	1.25	1.25	-	-
	C	-	1.00	4.25	4.00	4.25	3.50	2.50	1.00	-	-	-	-
ED6	A	-	-	1.75	2.00	3.50	4.00	0.75	-	-	-	-	-
	B	-	0.75	2.75	2.75	3.50	4.00	2.75	-	-	-	-	-
	C	-	1.00	4.75	2.50	3.50	3.00	2.25	1.75	-	-	-	-

Notes: (a) Samples from the control (sham-inoculated) calves were all negative
(b) Samples from all calves on days 12-15 were negative

APPENDIX 5(iv)

Virus titre (log_{10} $TCID_{50}$/90µl extraction medium) in left eye swabs (experiment 1)

Virus	Replicate	Days after inoculation											
		0	1	2	3	4	5	6	7	8	9	10	11
ED1	A	-	2.75	4.75	3.75	3.25	3.50	2.25	-	-	0.75	-	-
	B	-	1.25	4.50	5.50	-	3.00	1.00	1.50	-	-	-	-
	C	-	2.25	4.75	3.50	3.00	2.00	1.75	0.50	-	-	-	-
ED2	A	-	2.25	2.75	1.50	-	1.50	0.75	-	-	-	-	-
	B	-	-	-	3.25	-	4.00	0.75	-	-	-	-	-
	C	-	2.00	3.75	2.50	1.75	-	-	-	-	-	-	-
ED3	A	-	2.75	5.00	5.00	2.00	2.50	5.75	1.75	1.50	1.00	-	-
	B	-	-	1.00	3.50	1.00	3.50	1.75	-	0.75	-	-	-
	C	-	1.50	4.75	3.75	2.75	3.25	3.00	-	-	-	-	-
ED4	A	-	3.75	4.50	3.50	3.25	3.25	2.00	0.75	-	-	-	-
	B	-	2.00	-	-	2.75	4.25	2.25	1.75	0.75	-	-	-
	C	-	3.00	5.50	3.50	2.75	2.75	4.00	0.50	1.25	1.25	-	-
ED5	A	-	3.25	5.75	4.00	2.50	2.75	2.75	1.25	0.75	-	-	-
	B	-	-	2.50	3.25	3.75	4.00	-	1.00	-	-	-	-
	C	-	0.50	3.25	2.50	2.75	3.50	2.75	-	-	0.50	-	-
ED6	A	-	-	0.75	-	1.75	2.00	-	-	-	-	-	-
	B	-	1.25	4.25	2.50	1.50	3.00	-	0.75	-	-	-	-
	C	-	-	-	1.25	1.75	1.50	1.75	0.50	-	-	-	-

APPENDIX 5(v)

Virus titre (log_{10} $TCID_{50}$/90µl extraction medium) in right eye swabs (experiment 1)

Virus	Replicate	Days after inoculation											
		0	1	2	3	4	5	6	7	8	9	10	11
ED1	B	-	-	1.00	3.75	1.25	3.25	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-
ED2	B	-	-	-	-	-	1.75	-	-	-	-	-	-
	C	-	-	-	1.50	-	-	-	-	-	-	-	-
ED3	B	-	-	-	-	-	1.25	-	-	0.75	-	-	-
	C	-	-	-	0.50	1.00	1.00	3.25	1.00	-	1.00	-	-
ED4	B	-	-	-	-	1.75	1.75	-	-	-	-	0.75	-
	C	-	-	-	-	-	1.50	1.50	-	1.25	-	-	-
ED5	B	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	0.50	-	-	-	-	-	-	-	-	-
ED6	B	-	-	-	1.50	-	1.50	-	-	-	-	-	-
	C	-	-	0.50	-	-	-	-	-	-	-	-	-

Note: Right eye samples were not taken in replicate A.

APPENDIX 5(vi)

Virus titre (log_{10} $TCID_{50}$/90µl extraction medium) in eye swabs (Experiment 2)

Eye	Pair	Virus	Days after inoculation															
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
L	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	0.75	-	0.45	-	-	-	-	-
	5	1	-	-	-	-	-	1.50	2.75	3.00	2.25	2.75	-	-	-	-	-	-
		2	-	-	-	-	-	2.75	2.75	2.25	2.50	0.75	-	-	-	-	-	-
	2	1	-	-	-	-	-	1.50	3.50	1.50	-	-	-	-	-	-	-	-
		2	-	-	1.25	1.00	1.50	2.50	2.25	0.45	-	-	-	-	-	-	-	-
	3	1	-	-	-	2.25	2.25	2.25	2.50	2.50	0.75	-	-	-	-	-	-	-
		2	-	1.00	1.25	1.25	2.25	2.25	1.50	0.30	-	-	-	-	-	-	-	-
	4	1	-	-	-	-	1.75	2.00	4.25	1.75	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	0.75	1.50	0.75	-	-	-	-	-	-	-	-
	6	1	-	-	-	2.50	1.25	1.50	2.50	1.75	0.75	-	-	-	-	-	-	-
		2	-	-	-	0.75	1.75	1.00	2.25	1.75	-	-	-	-	-	-	-	-
R	1	1	-	-	-	-	-	-	1.75	2.50	0.30	0.75	0.30	-	-	-	-	-
		2	-	-	-	-	-	-	-	0.15	-	-	0.75	-	-	-	-	-
	5	1	-	-	-	-	0.15	1.00	3.75	3.00	3.00	2.50	0.75	0.60	0.30	-	-	-
		2	-	-	-	-	-	-	-	1.75	0.60	-	-	-	-	-	-	-
	2	1	-	-	-	-	-	-	0.15	0.45	1.00	-	-	-	-	-	-	-
		2	-	-	-	-	-	0.30	-	0.60	-	-	-	-	-	-	-	-
	3	1	-	-	-	-	-	-	-	2.25	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	1.50	-	-	-	-	-	-	-	-	-
	4	1	-	-	-	-	-	1.75	2.00	2.25	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	0.30	0.75	1.25	0.60	0.60	-	-	-	-	-
	6	1	-	-	-	-	-	1.00	3.50	1.00	0.15	0.45	-	-	-	-	-	-
		2	-	-	-	-	-	0.30	0.75	1.50	-	-	-	-	-	-	-	-

APPENDIX 5(vii)

Antibody responses to BHV1 in experiment 1A as measured by ELISA
(expressed as OD values)

Antibody class	Viral inoculum	Days after inoculation						
		0	3	7	10	14	21	28
IgG1	ED1	.13	.14	.11	.27	.62	.90	1.05
	ED2	1.05	nd	nd	.99	.90	.80	.92
	ED3	.11	.16	.16	.24	.51	.82	.96
	ED4	.11	.03	.04	.18	.65	.82	.87
	ED5	.13	.12	.13	.26	.75	.92	.92
	ED6	.11	.10	.08	.11	.30	.47	.70
	control	.16	nd	nd	nd	.13	nd	.11
	control	.09	nd	nd	nd	nd	nd	.05
IgG2	ED1	.02	.02	.02	.06	.21	.39	.49
	ED2	.13	nd	nd	.14	.13	.13	.15
	ED3	.01	.01	.01	.05	.17	.36	.58
	ED4	0	0	0	.04	.19	.26	.31
	ED5	.02	.01	.01	.05	.27	.37	.41
	ED6	.01	.01	.01	.02	.09	.16	.23
	control	0	nd	nd	nd	.01	nd	0
	control	.09	nd	nd	nd	nd	nd	0
IgM	ED1	.09	.08	.13	.95	.73	.26	.12
	ED2	.27	nd	nd	.19	.15	.09	.08
	ED3	.05	.13	.25	1.28	.98	.29	.16
	ED4	.03	.06	.18	.98	.61	.17	.09
	ED5	.06	.06	.08	.79	.38	.12	.08
	ED6	.13	.09	.09	.25	.28	.13	.12
	control	.09	nd	nd	nd	.05	nd	.02
	control	.05	nd	nd	nd	nd	nd	.04

APPENDIX 5(viii)

Antibody responses to BHV1 in experiment 1B as measured by ELISA
(expressed as OD values)

Antibody class	Viral inoculum	Days after inoculation						
		0	7	11	22	30	45	51
IgG1	ED1	.04	.04	.29	.76	1.05	.57	.32
	ED2	.12	10	.25	.73	.95	1.26	1.32
	ED3	.02	.01	.13	.77	1.35	1.30	1.29
	ED4	.07	.06	.20	.74	.93	.95	.99
	ED5	.02	.03	.22	.90	1.02	1.01	.92
	ED6	.18	.11	.21	.47	.63	.74	.85
	control	.26	.22	.17	.14	.15	.10	.11
IgG2	ED1	.01	.01	.04	.10	.16	.40	.45
	ED2	.01	.01	.08	.35	.62	1.09	1.08
	ED3	.01	.01	.02	.13	.40	.53	.66
	ED4	0	.01	.04	.31	.47	.64	.69
	ED5	.01	.01	.07	.34	.48	.60	.55
	ED6	.01	.01	.04	.15	.23	.33	.43
	control	.05	.04	.04	.02	.01	.01	.01
IgM	ED1	.07	.10	1.17	.11	.06	0	0
	ED2	.09	.07	.64	.19	.11	.34	.33
	ED3	.02	.08	.93	.31	.13	.15	.13
	ED4	.17	.15	.74	.11	.11	.15	.19
	ED5	.07	.11	.43	.11	.11	.15	.14
	ED6	.16	.16	.64	.18	.16	.21	.23
	control	.11	.09	.09	.15	.16	.11	.18

APPENDIX 5(ix)

Antibody responses to BHV1 in experiment 1C as measured by ELISA
(expressed as OD values)

Antibody class	Viral inoculum	Days after inoculation															
		0	2	4	6	7	8	9	10	12	14	16	19	21	28	42	56
IgG1	ED1	.13	.11	.10	.08	.10	.08	.07	.13	.18	.54	.78	.89	1.01	1.00	1.08	1.15
	ED2	.03	.01	.02	.02	.02	.05	.06	.11	.21	.31	.34	.35	.36	.44	.62	.75
	ED3	.02	.01	0	.01	.01	.04	.09	.19	.37	.57	.72	.85	.94	1.05	1.21	1.44
	ED4	.09	.09	.09	.08	.09	.09	.13	.18	.34	.50	.79	.97	1.12	1.16	1.26	1.28
	ED5	.04	.04	.04	.06	.08	.07	.10	.16	.37	.80	.91	.92	.93	1.01	1.08	1.13
	ED6	.02	.02	.03	.02	.03	.04	.08	.14	.32	.50	.51	.62	.64	1.04	1.07	1.23
	control	.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	.02	nd
IgG2	ED1	.01	.01	.01	.01	.01	.01	.01	.02	.03	.11	.19	.26	.28	.28	.34	.45
	ED2	.01	0	.01	.01	.01	.01	.01	.03	.06	.13	.16	.18	.18	.22	.29	.34
	ED3	0	0	0	0	0	.01	.01	.04	.08	.17	.27	.38	.46	.59	.81	1.09
	ED4	0	.01	.01	.01	.02	.02	.02	.04	.09	.14	.31	.47	.55	.65	.91	1.05
	ED5	.01	.01	.01	.01	.01	.01	.02	.02	.09	.27	.38	.39	.40	.45	.60	.70
	ED6	.01	.01	01	.01	.01	.01	.02	.03	.10	.16	.17	.19	.16	.33	.36	.47
	control	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	.01	nd
IgM	ED1	.08	.08	.08	.09	.10	.11	.19	.42	.46	.38	.31	.19	.14	.12	.15	.20
	ED2	.18	.05	.15	.14	.15	.23	.36	.52	.56	.44	.34	.22	.17	.21	.14	.18
	ED3	.05	.05	.04	.08	.09	.30	.72	1.17	1.36	1.01	.81	.40	.34	.19	.17	.27
	ED4	.07	.11	.09	.13	.14	.28	.59	1.06	1.37	.90	.65	.25	.28	.15	.16	.23
	ED5	.04	.06	.06	.09	.12	.14	.35	.45	.50	.43	.32	.21	.18	.17	.18	.22
	ED6	.11	.14	.17	.17	.15	.21	.43	.55	.46	.37	.22	.13	.10	.15	.14	.18
	control	.06	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	.14	nd

APPENDIX 5(x)

IgG1 antibody responses in experiment 2
(expressed as OD values)

Pair	Treatment*	Virus strain	Days after inoculation																
			0	7	11	14	21	32	42	56	63	70	88	92	95	98	102	111	130	187
1	C	ED1	.16	.16	.19	.64	1.13	1.29	1.34	1.55		1.48	1.51							1.66
	C	ED2	.12	.14	.09	.26	1.11	1.20	1.23	1.11		1.30	1.00							1.12
5	C	ED1	0	0	.07	.56	1.73	2.18	2.30	2.55		2.57	2.43							2.24
	C	ED2	0	.07	.10	.49	.97	1.33	1.63	1.68		1.96	1.97							1.77
2	IH	ED1	.09	.05	.43	.99	1.23	1.31	1.42	1.34		1.10	1.22	1.19	1.79	1.99	1.97	1.99	1.75	1.40
	IH	ED2	0	.04	.16	.44	.46	.66	.82	.80		1.00	1.04	.92	1.36	1.76	1.94	1.92	1.78	1.34
3	IH	ED1	0	0	.08	.28	.46	.57	.71	.77		.94	.84	.77	1.08	1.23	1.40	1.43	1.23	1.12
	IH	ED2	.01	.01	.17	.48	.74	.87	.88	.92		.99	.83	.77	1.15	1.55	1.79	2.02	1.93	1.34
4	ID	ED1	.06	.04	.28	.70	1.34	1.56	1.67	1.65	1.50	2.37	2.37							1.42
	ID	ED2	.07	.09	.30	.54	.85	1.03	1.21	1.28	1.28	2.35	2.34							‹
6	I	ED1	.09	.09	.34	.66	.97	1.29	1.50	1.43		1.55	1.45							1.67
	ID	ED2	.01	0	.12	.42	.91	1.30	1.27	1.43	1.31	1.59	1.36							.82

Notes: * Treatments: I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

‹ Calf died before this date

APPENDIX 5(xi)

IgG2 antibody responses in experiment 2
(expressed as OD values)

Pair	Treatment*	Virus strain	Days after inoculation																
			0	7	11	14	21	32	42	56	63	70	88	92	95	98	102	111	130	187
1	C	ED1	.05	.04	.05	.17	.45	.61	.74	.93		1.03	.85							.99
	C	ED2	.07	.07	.05	.07	.31	.42	.46	.43		.68	.37							.56
5	C	ED1	0	0	.02	.14	.51	.92	1.12	1.95		2.05	1.70							1.66
	C	ED2	0	0	.02	.11	.41	.46	.67	.67		.92	.85							.92
2	IH	ED1	.04	.01	.07	.24	.31	.43	.49	.68		.47	.73	.72	.82	1.16	1.33	1.37	1.21	1.49
	IH	ED2	0	.04	.08	.22	.32	.56	.64	.55		.84	.89	.77	.86	1.27	1.67	1.82	2.00	1.64
3	IH	ED1	.01	.06	.08	.30	.44	.58	.81	.99		1.19	1.18	1.05	1.16	1.47	1.62	1.64	1.51	1.29
	IH	ED2	.01	.05	.23	.40	.59	.76	.98	1.30		1.30	1.25	1.13	1.19	1.76	2.04	2.19	2.26	2.04
4	ID	ED1	0	0	.06	.19	.47	.68	.89	.95	1.05	2.04	2.04							1.06
	ID	ED2	0	.02	.08	.16	.21	.35	.48	.58	.69	1.69	1.67							‹
6	I	ED1	0	0	.07	.21	.37	.73	.87	.97		1.01	.92							.92
	ID	ED2	0	0	.05	.24	.40	.64	.64	.87	.79	1.14	1.21							.74

Notes: * Treatments: I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

‹ Calf died before this date

APPENDIX 5(xii)

IgM antibody responses in experiment 2
(expressed as OD values)

Pair	Treatment*	Virus strain	Days after inoculation																
			0	7	11	14	21	32	42	56	63	70	88	92	95	98	102	111	130	187
1	C	ED1	.33	.25	.60	.75	.45	.26	.20	.12		.26	.40							.22
	C	ED2	.08	.06	.13	.89	.63	.20	.19	.07		0	.01							.15
5	C	ED1	0	0	0	.62	.32	.22	.18	.34		.40	.20							.16
	C	ED2	.02	.04	.08	.70	.34	.14	.04	.10		.20	.16							.04
2	IH	ED1	.04	.08	.84	.50	.21	.07	.05	.04		.13	.13	.03	.14	.06	.18	.20	.04	.11
	IH	ED2	0	0	.93	.61	.20	.13	.15	.13		.20	.19	.17	.19	.37	.44	.41	.43	.24
3	IH	ED1	0	0	.86	.63	.19	.07	.05	.05		.08	.04	0	.05	.07	.11	.11	.04	.05
	IH	ED2	0	.03	.30	.12	.10	.03	.08	.07		.11	.05	.12	.15	.07	0	.19	0	0
4	ID	ED1	0	0	.74	.44	.14	.04	0	0	.02	.18	.20							0
	ID	ED2	0	0	.56	.44	.12	0	0	.04	0	.14	.20							‹
6	I	ED1	0	0	1.04	.58	.14	.02	.06	.12		.10	.12							.12
	ID	ED2	0	0	0	.04	.06	.02	.04	.08	.04	.14	.10							.02

Notes: * Treatments: I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

‹ Calf died before this date

APPENDIX 5(xiii)

Antibodies to BHV1 in nasal mucus measured by virus neutralization (VN) (end point titre) or immunoglobulin class-specific ELISA (OD values, for mucus diluted 1/20), Experiment 1C

Antibody	Virus	Days after inoculation 1	5	8	12	16	21
VN	ED1	<1	<1	<2	1	3	3
	ED2	<1	<4	<4	<4	2	1.5
	ED3	<1	<1	<1	5	>8	3
	ED4	<1	<1	<1	3	6	3
	ED5	<1	<1	<1	<1	1	1.5
	ED6	<1	<1	<1	1	1.5	2
IgM	ED1	.02	.01	.07	.81	1.02	.25
	ED2	.02	.02	.12	.42	.32	.20
	ED3	0	.04	.18	1.79	1.02	.36
	ED4	.01	0	.14	1.58	.99	.50
	ED5	.01	0	.12	.50	.31	.14
	ED6	.01	.01	.32	.62	.42	.20
IgA	ED1	.03	.01	.02	.41	.34	.24
	ED2	.04	.01	.03	.10	.09	.07
	ED3	.02	0	.06	.28	.23	.41
	ED4	.02	.01	.03	.28	.46	.76
	ED5	.02	.01	.02	.21	.07	.19
	ED6	0	.01	.06	.40	.31	.10
IgG1	ED1	.03	.02	.02	.20	.85	.77
	ED2	.02	.02	.03	.12	.26	.41
	ED3	.02	.02	.03	.51	.71	.69
	ED4	.02	.02	.04	.20	.67	.78
	ED5	.02	.02	.03	.29	.64	.48
	ED6	.01	.02	.03	.20	.46	.62
IgG2	ED1	.04	.01	.01	.17	.91	.65
	ED2	.02	.03	0	.08	.26	.49
	ED3	.02	0	.02	.30	.73	.66
	ED4	.01	.01	.01	.26	.92	.90
	ED5	.03	.02	.01	.25	.71	.62
	ED6	0	.01	.02	.22	.49	.57

APPENDIX 5(xiv)

IgG1 antibodies detected by ELISA (OD values) in nasal mucus in experiment 2

Pair	Treatment*	Virus Strain	Days after inoculation									
			0	14	21	32	42	56	63	70	88	102
1	C	ED1	.06	.23	.63	.91	.76	1.00		.68	.65	
	C	ED2	.03	.19	.81	.66	.34	.41		.34	.29	
5	C	ED1	.03	.18	.79	.85	.96	1.23		.80	1.29	
	C	ED2	.01	.14	.42	.67	.44	.69		.44	.59	
2	IH	ED1	.03	.86	1.00	.72	1.05	.72		.52	.38	1.16
	IH	ED2	.06	.47	.73	.91	.93	1.03		.42	.74	1.16
3	IH	ED1	.03	.30	.40	.27	.32	.68		.97	.69	1.28
	IH	ED2	.04	.44	.47	.35	.48	.54		.33	.37	.92
4	ID	ED1	.03	.51	.52	.46	.29	.53	.67	1.16	1.19	
	ID	ED2	.15	.40	.34	.42	.40	.85	.66	1.49	1.34	
6	I	ED1	.02	.53	.69	.61	.72	.82		.67	.57	
	ID	ED2	0	.25	.44	.47	.45	.63	.35	.59	.42	

* Treatments: I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

APPENDIX 5(xv)

IgG2 antibodies detected by ELISA (OD values) in nasal mucus in experiment 2

Pair	Treatment*	Virus strain	Days after inoculation									
			0	14	21	32	42	56	63	70	88	102
1	C	ED1	.04	.13	.38	.72	.68	.96		.69	.56	
	C	ED2	.05	.09	.31	.34	.23	.33		.25	0	
5	C	ED1	.01	.09	.36	.61	.76	1.04		.99	1.11	
	C	ED2	0	.07	.26	.46	.32	.46		.36	.49	
2	IH	ED1	.01	.32	.36	.34	.57	.48		.43	.39	1.18
	IH	ED2	.03	.19	.32	.41	.44	.49		.20	.42	.85
3	IH	ED1	0	.22	.33	.27	.38	.93		1.14	.91	1.29
	IH	ED2	.03	.36	.38	.34	.48	.60		.36	.43	.90
4	ID	ED1	0	.26	.31	.38	.29	.65	.89	1.25	1.28	
	ID	ED2	.01	.17	.20	.29	.27	.60	.48	1.02	1.06	
5	I	ED1	.02	.32	.43	.53	.70	.88		.81	.60	
	ID	ED2	.01	.25	.33	.47	.47	.73	.48	.79	.56	

* Treatment I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

APPENDIX 5(xvi)

IgM antibodies detected by ELISA (OD values) in nasal mucus in experiment 2

Pair	Treatment*	Virus Strain	Days after inoculation									
			0	14	21	32	42	56	63	70	88	102
1	C	ED1	.08	.51	.22	.20	.16	.18		.14	.08	
	C	ED2	.02	1.15	.39	.14	.46	.15		.05	.06	
5	C	ED1	.10	.60	.27	.19	.22	.34		.18	.35	
	C	ED2	.02	.69	.28	.19	.28	.17		.06	.09	
2	IH	ED1	.04	.69	.34	.14	.15	.09		.09	.04	.38
	IH	ED2	.05	.53	.29	.24	.24	.24		.06	.12	.34
3	IH	ED1	.03	.50	.17	.09	.11	.15		.17	.13	.37
	IH	ED2	.04	.28	.10	.07	.10	.07		.08	.10	.36
4	ID	ED1	.01	.65	.19	.06	.04	.09	.13	.37	.18	
	ID	ED2	.05	.64	.19	.10	.06	.12	.11	.45	.19	
6	I	ED1	.02	.63	.21	.14	.15	.18		.18	.12	
	ID	ED2	0	.34	.12	.14	.09	.19	.11	.22	.11	

* Treatments: I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

APPENDIX 5(xvii) IgA antibodies detected by ELISA (OD values) in nasal mucus in experiment 2

Pair	Treatment*	Virus strain	Days after inoculation									
			0	14	21	32	42	56	63	70	88	102
1	C	ED1	.02	.08	.04	.03	0	0		.03	0	
	C	ED2	0	.15	.07	.05	.06	.04		.06	.04	
5	C	ED1	.03	.12	.05	.10	.13	.04		.06	.07	
	C	ED2	.09	.09	.08	.10	0	.11		.05	.09	
2	IH	ED1	.02	.19	.06	.08	0	.02		.06	.13	.41
	IH	ED2	.01	.13	.05	.04	.02	0		.03	.04	0
3	IH	ED1	0	.26	.08	.14	.09	.08		.03	.04	.74
	IH	ED2	0	.07	.06	.06	.05	.10		.10	.04	.50
4	ID	ED1	0	.08	.11	.09	.05	.04	.07	.61	.51	
	ID	ED2	.03	.09	.06	.30	.12	.08	.06	.22	.15	
6	I	ED1	.02	.13	.09	.09	.06	.09		.17	.09	
	ID	ED2	0	.03	.05	.07	.16	.21	.15	.30	.23	

* Treatments: I = inoculated on day 0
C = in-contact from day 0
D = dexamethasone on days 56-60 inclusive
H = heterologous virus strain challenge on day 88

APPENDIX 6 (i) Results of virus isolation, amplified ELISA and reverse passive haemagglutination for BHV1 antigen on swab extracts from BHV1 - vaccinated calves in experiment 5

Days after challenge	Test (a)	Calf No. 35	58	88	41	54	44	68	2	43	47
2	V	2.25	3.5	1.25	2.5	3.0	0.75	-	-	-	1.75
	E	-	-	-	0.03	0.02	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	2
3	V	1.0	3.25	1.25	3.5	2.75	0.75	2.0	-	3.25	-
	E	-	-	-	0.02	0.01	-	-	-	0.02	-
	R	-	-	1	-	-	-	-	1	-	2
4	V	3.25	2.5	0.75	5.0	4.25	0.75	0.75	0.75	1.75	1.0
	E	-	0.05	-	0.22	0.03	-	-	-	-	-
	R	-	2	2	6	1	1	NA	2	-	2
6	V	1.5	1.5	-	4.5	-	1.75	1.25	2.0	3.5	1.0
	E	-	-	-	0.07	0.01	-	0.04	-	-	-
	R	-	-	-	2	-	-	-	-	2	-
7	V	0.75	-	-	3.0	-	3.0	1.25	-	-	1.5
	E	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	2
8	V	-	-	-	0.75	-	-	-	0.75	-	-
	E	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	2	1	2	-	-
9	V	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	1
10	V	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	-

Notes (a) V = Virus isolation, $\log_{10}$ $TCID_{50}$/90 µl

E = BAB ELISA, optical density (test well - control well).

R = RPHA, $\log_2$ end-point titre

- = no virus isolated, or OD ≤ 0, or no haemagglutination observed for V, E and R respectively.

APPENDIX 6 (ii) Results of virus isolation, amplified ELISA and reverse passive haemagglutination for BHV1 antigen on swab extracts from non-vaccinated calves in experiment 5.

Days after challenge	Test[a]	Calf No. 14	48	70	1	62	74	96	4	16	63	89	64	82	84	Mean [b]
2	V	0.75	1.25	3.75	2.5	3.0	4.5	3.75	1.0	3.5	3.25	2.5	1.75	1.75	3.0	2.56
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	R	1	–	1	–	–	–	1	–	–	1	–	–	–	–	0.3
3	V	3.0	4.5	3.5	4.0	2.5	3.0	3.5	2.5	3.25	3.75	5.25	2.75	3.75	4.5	3.63
	E	–	0.10	0.11	0.02	–	0.06	0.05	–	0.09	–	0.20	–	–	0.13	0.05
	R	1	2	2	3	–	1	1	–	1	–	1	–	–	1	1.0
4	V	5.0	4.75	4.75	4.25	5.75	3.75	5.25	2.75	4.5	3.0	5.0	3.75	4.5	4.75	4.31
	E	0.27	0.21	1.03	0.30	0.50	0.19	0.98	0.70	0.82	0.18	0.38	0.09	0.13	0.69	0.46
	R	6	4	8	5	6	3	7	5	5	3	3	3	1	5	4.1
6	V	5.0	3.25	3.5	4.75	2.0	4.5	4.5	3.25	3.5	5.0	4.5	4.5	5.0	5.75	4.24
	E	0.48	0.50	0.25	0.27	–	NA	NA	0.41	0.12	0.30	0.32	0.55	0.46	0.56	0.35
	R	6	6	4	5	–	1	NA	3	1	3	3	5	3	3	3.6
7	V	4.0	4.25	2.5	1.25	0.75	3.0	2.75	3.5	3.0	3.5	3.5	4.75	3.75	5.0	3.44
	E	–	0.13	0.07	–	–	–	0.06	0.07	0.09	0.08	0.28	0.30	–	0.28	0.10
	R	1	2	2	–	–	–	1	1	1	1	3	3	–	3	1.5
8	V	–	3.0	2.25	1.25	–	1.5	2.25	1.5	2.5	1.5	2.5	3.25	4.25	3.25	2.23
	E	–	–	–	–	–	–	–	–	–	–	0.06	–	–	0.08	0.01
	R	–	2	–	1	1	–	–	–	–	–	1	1	–	1	0.4
9	V	1.5	1.5	1.5	–	–	0.75	2.0	–	0.75	0.75	2.0	1.75	2.5	1.5	1.27
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	R	–	–	–	–	–	1	–	–	–	–	–	–	–	–	0.1
10	V	–	–	–	0.75	–	–	0.75	1.0	–	–	1.0	0.75	1.0	1.0	0.48
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	R	–	1	–	–	–	–	–	–	–	–	–	–	–	–	0.1

Notes: (a) V = Virus isolation, $\log_{10}$ $TCID_{50}$/90 µl.
E = BAB ELISA, optical density.
R = RPHA, $\log_2$ end point titre
(b) Excluding animal 62.

Papers and Articles

Changing trends in infectious bovine rhinotracheitis in Great Britain

S. Edwards

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The incidence and severity of infectious bovine rhinotracheitis in Great Britain increased markedly in the mid to late 1970s. Data available in MAFF records have been used to analyse the changing trends in disease incidence and serological prevalence, with particular emphasis on the most recent decade.

EPIDEMIOLOGICAL data on infectious bovine rhinotracheitis (IBR) and related diseases in Great Britain are incomplete. Early papers were presented as case reports of IBR (Dawson and others 1962, Darbyshire and Shanks 1963) and infectious pustular vulvovaginitis/balanoposthitis (Huck and others 1971, Collings and others 1972). Serological surveys gave an estimated seroprevalence for the virus as 2·1 per cent (Dawson and Darbyshire 1964), 6·8 per cent (sampled in 1974) (Kirby and others 1978) and 12 per cent (sampled in 1977) (D. H. Roberts personal communication, summarised by Anon 1978); however, these three studies used different methods of antibody assay, virus neutralisation in tube cultures, indirect haemagglutination and virus neutralisation in microplates, respectively.

Beginning in the winter of 1977 to 1978, widespread reports (summarised by Anon 1979) indicated a sudden rise in the incidence and clinical severity of IBR, typically associated with high herd morbidity and variable but significant mortality. The evidence for this rise in incidence was supported by clinical and pathological studies by Wiseman and others (1978, 1980) and Allan and others (1980). Subsequently, Msolla and others (1981) suggested that the seroprevalence of bovine herpesvirus 1 (BHV1) in Scotland had increased to 12 per cent, notably in herds which had introduced Holstein cattle. They did not state the year in which the samples were collected. A similar prevalence (11·4 per cent) was reported by Peters and Perry (1983) for bulls at performance testing stations in the period 1979 to 1981. Further developments, likely to influence the epidemiology of the disease, occurred in 1979 and 1981 with the announcement of product licences and the commercial availability of the live attenuated intranasal IBR vaccines Tracherine (Smith Kline Animal Health) and Nasalgen (Wellcome).

In an attempt to gain an overall view, particularly for the period 1970 to 1986, data from the records of the veterinary laboratories of the Ministry of Agriculture, Fisheries and Food (MAFF) and the Scottish Agricultural Colleges have been further analysed.

Materials and methods

Three sources of data were used. None represents an unbiased sample of the cattle population and two were derived from the same source material – namely clinical incidents presented to the veterinary investigation (VI) service for disease investigation and diagnostic laboratory testing.

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VIDA II

The veterinary investigation diagnosis analysis – Mark II (VIDA II) commenced in 1975 and comprised over 2·2 million computerised records by the end of 1986 (MAFF and others 1987). The data were supplied by VI centres in England, Scotland and Wales.

Diseases caused by bovine herpesvirus 1 (BHV1) were not recorded as specific diagnoses on VIDA II until 1980, when they appeared under 'Group 1: Systemic diseases of cattle and those not readily classified organically' as 'IBR/infectious pustular vulvovaginitis (IPV)' and 'Group 9: Diseases of the reproductive and mammary systems of cattle' as 'Fetopathy due to IBR/IPV'. The data were analysed by month and year and by county or region.

Central Veterinary Laboratory diagnostic testing

Materials for diagnostic testing were received at the virology department, Central Veterinary Laboratory, Weybridge (CVL) via the VI service. All incidents investigated at the CVL were therefore also recorded on VIDA. Nevertheless, more detailed records were available at the CVL than were entered on VIDA and a separate analysis was carried out. Conversely, not all VIDA incidents were investigated at the CVL – during the study period, an increasing number of diagnostic tests for BHV1 were carried out in VI centres without further referral, while in Scotland most tests were carried out at the Moredun Institute in Edinburgh. Nevertheless, sufficient testing was done at the CVL to warrant analysis of the data.

Information on the number of tests done and number of positive diagnoses was available, through the virology department's annual reports, from 1969. In addition, from 1977 onwards detailed case records were kept of all incidents in which a diagnosis of active BHV1 infection was considered to have been established, either by virus isolation and antigen detection or by seroconversion. These records included the laboratory reference numbers, the date, the VI centre of origin, the basis of the laboratory diagnosis, and as much of the clinical and pathological history as was supplied on the sample submission document. The history varied from nothing to a full, detailed account of the incident. The data in these case records were subsequently reduced to a codified form and entered into a computer database for analysis.

CVL, virology department statutory and export testing

Serum samples were submitted directly to the CVL by veterinary practitioners and by artificial insemination (AI) organisations for screening for BHV1 antibodies. The samples were from healthy animals, either before export or before entry to AI studs. The data were not recorded on VIDA and represented a section of the cattle population different from

case there were milking machine defects but in the other instance the problem remained undiagnosed.

Sheep

Nervous system diseases

Diagnoses of cerebrocortical necrosis were made at Auchincruive, Dumfries and Edinburgh. Dumfries and St Boswells also saw listeriosis.

At Dumfries three ewes were submitted from a farm where signs of ill-thrift, weight loss, skin irritation and nervous signs had been noted. Scrapie was suspected and post mortem examination of a South Country Cheviot gimmer was diagnosed on brain histopathology as suffering from scrapie. One other ewe was found to be hypomagnesaemic and another was confirmed as having cerebrocortical necrosis. Other ewes were examined which were showing signs of pruritus and orf and *Dermatophilus* species was recovered. It would appear that several different conditions were contributing to the problem.

On another farm, a four-year-old greyface ewe showing central nervous system signs, a suppurative meningeal encephalitis and choroiditis indicative of bacterial infection was diagnosed. *P haemolytica* was also recovered in septicaemic form and a necrotic suppurative pneumonia was also present.

Generalised and systemic diseases

All centres commented on occasional incidents associated with low levels of trace elements while Edinburgh also saw copper poisoning. Sporadic cases of enterotoxaemia and pasteurellosis were also reported.

Alimentary system diseases Parasitic gastroenteritis was diagnosed at all centres. Ayr saw a four-month-old lamb with a worm egg count of 9700/g. Perth reported a few cases of coccidiosis. Thurso saw 12 lambs which died as a result of damage caused by the use of a dosing gun. A further similar case was reported from Aberdeen. There were two cases of Johne's disease from Shetland.

Respiratory system diseases A few cases of *Pasteurella haemolytica* pneumonia were seen at Aberdeen and Perth. Single cases of pulmonary adenomatosis were reported from Perth and Ayr. Ayr saw several cases of pneumonic pasteurellosis. Dumfries saw pneumonia and purulent pleurisy in blackface ewes associated with *C pyogenes*.

Musculoskeletal system diseases *Erysipelas rhusiopathiae* was isolated from the joint of a stiff lamb where problems have been occurring with stiffness and lameness in four-month-old lambs. A serological response of 1/5120 was also noted from this animal.

Reproductive system diseases Ayr, Edinburgh and Dumfries commented upon various abnormalities found during tup testing.

Pigs

At St Boswells *Mycoplasma hyosynoviae* was identified as the cause of joint swellings in a minimal disease pig unit. Serological examination of gilts at entry to the unit and two weeks later revealed high titres to *M hyosynoviae* with no evidence of seroconversion. It was concluded that infection was well established in the gilts before arrival on the unit.

An outbreak of red gut was seen in a 12 sow unit where two of 28 three-month-old pigs died suddenly. No satisfactory dietary cause was identified. The condition had not been seen before on the farm.

Perth saw streptococcal septicaemia in young pigs and *Pasteurella multocida* pneumonia in a fattening pig.

Birds

Gamebirds

Gamebird submissions constituted the bulk of avian work during August. At Dumfries losses occurred mainly among eight-week-old pheasants from a variety of sources. All were found to be badly feathered along backs and wings due to severe feather picking, and often there was a lack of food in the intestinal tract. While *Escherichia coli* was recovered in septicaemic form, it seemed likely that stress factors including marble spleen disease were contributing to deaths in these situations.

The most serious problem encountered at Edinburgh was an almost complete wipe out – mainly over a weekend – of 10-to-11-week-old pheasants in one release pen. Yersiniasis was the principal diagnosis.

At St Boswells *Heterakis isolonche* infestation associated with histomoniasis was seen in a batch of pheasants. This worm, which is far more pathogenic than *H gallinarum*, was seen despite earlier successful treatment for *Syngamus trachea*.

There was a steady intake of pheasant material to Perth throughout August. Diagnosis included trichomoniasis (two), coccidiosis (three), rotavirus (one), colisepticaemia (one) and exposure (one). One bird had gapeworms, staphyloccal joint infection and a group E salmonella.

Mycotic air sacculitis was seen at Aberdeen.

Several batches of intestines were submitted from grouse on Perthshire moors. Very high worm burdens were present despite the use of medicated grit.

Aberdeen isolated *Yersinia pseudotuberculosis* in eight-week-old red legged partridges and diagnosed colisepticaemia in nine-week-old grey partridges.

Fowl Broiler ascites syndrome was reported from Thurso and Aberdeen. Coccidiosis was seen at Perth. Severe infestation with *Dermanyssus gallinarum* was the cause of death and reduced egg production in a small flock of free range hens seen at St Boswells. At the same centre *H gallinarum* was also seen in association with histomoniasis in hens.

Geese Perth saw gizzard worms as the cause of death of several birds on one unit.

Pigeons

Edinburgh found that submissions from racing pigeons continued at a moderate level with viral hepatitis being the diagnosis of the month in young birds which were scouring or going light. Trichomoniasis was also seen as a problem in young birds showing typical liver lesions.

Deaths among wild sparrows and pigeons in a Dundee factory caused concern. Post mortem examination revealed a miscellany of different, apparently unrelated conditions and it was concluded that the death represented normal wastage among a very large avian population in the area.

Paramyxovirus infection was diagnosed in unvaccinated doves in an aviary at Aberdeen.

Miscellaneous mammals

Strangles was confirmed in horses at Dumfries and St Boswells; Auchincruive and Edinburgh diagnosed ringworm in horses caused by *Trichophyton equinum* and *Microsporum equinum*, respectively. St Boswells saw *D arnfieldi* infestation in an adult Arab cross.

Edinburgh saw an outbreak of dermatophilus infection in goats.

Auchincruive isolated *Campylobacter jejuni* from a diarrhoeic pup.

Carbamate poisoning was confirmed by Edinburgh in two cats from an area where two other cats had also died in suspicious circumstances. Perth reported cases of ringworm (all *Microsporum canis*) involving two cats and a dog.

Cryptosporidiosis continued to cause deaths in red deer calves up to a week old on a unit in the Perth centre area.

TABLE 1: Annual number of herd incidents of IBR/IPV confirmed by laboratory tests

Year	CVL records: Seroconversion Respiratory disease	CVL records: Seroconversion Reproductive disease	CVL records: Virus isolation	VIDA II: Case records	VIDA II: Systemic disease	VIDA II: Fetopathy
1969	x	0	3	x	x	x
1970	x	1	3	x	x	x
1971	2	2	0	x	x	x
1972	5	0	1	x	x	x
1973	5	5	0	x	x	x
1974	2	3	0	x	x	x
1975	4	4	0	x	x	x
1976	11	8	1	x	x	x
1977	21	9	5	17	x	x
1978	41	10	30	81	x	x
1979	50	18	58	112	x	x
1980	26	17	73	107	517	14
1981	37	7	97	111	602	13
1982	44	13	92	111	705	30
1983	56	16	55	96	780	52
1984	45	7	44	85	631	72
1985	46	20	34	94	654	86
1986	31	18	17	74	483	85

x Data not available

the previous analyses, ie, predominantly pedigree breeding herds and, in the case of the AI samples, males only.

Two major changes in serological procedures for BHV1 diagnosis were made at the CVL during the study period, in each case involving an increase in test sensitivity and therefore the possibility that more seropositive animals would be detected. Until April 1976 the virus neutralisation test was carried out in test tube cell cultures using 0·1 ml per tube of serial serum dilutions mixed with an equal volume of medium containing 100 $TCID_{50}$ BHV1 as described by Dawson and others (1962). From April 1976 until August 1985 the virus neutralisation test was carried out in the microtitre plate system using 0·025 ml of serum dilution and 100 $TCID_{50}$ of virus per test well following the procedure described by Frerichs and others (1982). The method is still in use for most export tests. Some export tests and all AI and routine diagnostic tests have been performed by enzyme immunoassay (ELISA) since August 1985, as described by Hebert and others (1985) and Edwards and others (1986).

Statistical analysis

Frequency distributions of the incidence and prevalence of BHV1 infections were analysed by the χ^2 technique for one or two dimensional tables as appropriate.

Results

General

During the period 1980 to 1986, 4724 incidents of IBR/IPV were recorded on VIDA II, 4372 under the heading of systemic diseases and 352 as fetopathies. At the CVL, serological investigations for BHV1 were carried out on paired sera from 2656 incidents of respiratory disease in the period 1971 to 1986. Rising antibody titres to BHV1 were shown in 426 (16 per cent) of the incidents. Similar figures for reproductive disease for 1970 to 1985 were 2086 incidents investigated, of which 158 (7·6 per cent) had rising titres to BHV1. Submission of tissues and swabs for virus isolation could not be separated by disease category but BHV1 was isolated from 513 of the total 19,189 disease incidents investigated in the period 1969 to 1986. An unquantifiable proportion of these incidents were also investigated by serology and therefore included in both totals. Separate successive submissions of samples to the CVL from the same disease incident in any one herd could not be identified from the available data. Such submissions were therefore recorded as separate incidents for the CVL analysis although recorded only once in the VIDA II analysis. Evidence from the CVL case records analysis, for which this information was available, suggested that there was little over-reporting. The case record analysis included 888 BHV1 associated clinical incidents in the decade 1977 to 1986.

Testing of bulls for BHV1 antibody before entry to AI centres commenced in 1970. Up to the end of 1986, 26,585 animals were tested, of which 2716 (10·2 per cent) were positive. During the period 1969 to 1986, 61,257 cattle were tested for BHV1 antibody for export certification purposes and of these 5447 (8·9 per cent) were positive.

Temporal analysis

The number of herd incidents each year, extracted from the various record sources, is shown in Table 1. There is evidence of a marked increase in incidence in the late 1970s, and the beginnings of a decline in incidence in the mid-1980s. When the figures were expressed as a proportion of all incidents tested for IBR (CVL data) or of all cattle incidents (VIDA II data) the χ^2 test indicated a significant annual variation ($P<0·001$) for all categories except reproductive disease serology ($P>0·10$). The trends in proportional incidence are shown in Fig 1, in which year to year fluctuations have been smoothed by plotting three year rolling mean values.

The prevalence of serologically positive animals among healthy cattle (AI and export tests) also rose significantly

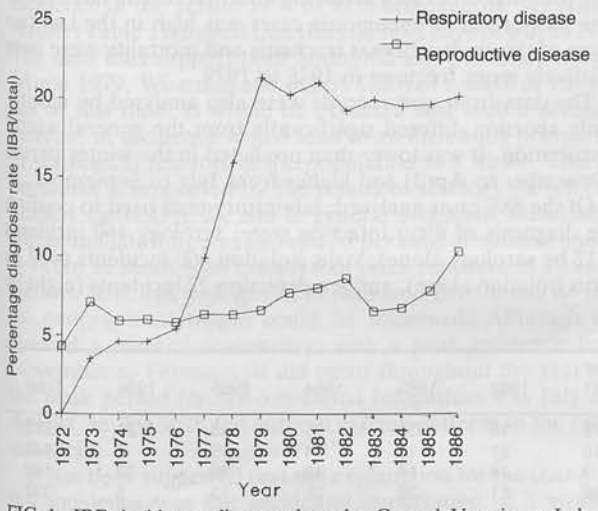

FIG 1: IBR incidence diagnosed at the Central Veterinary Laboratory. (Points on the graph represent the mean of the year shown and the two preceding years)

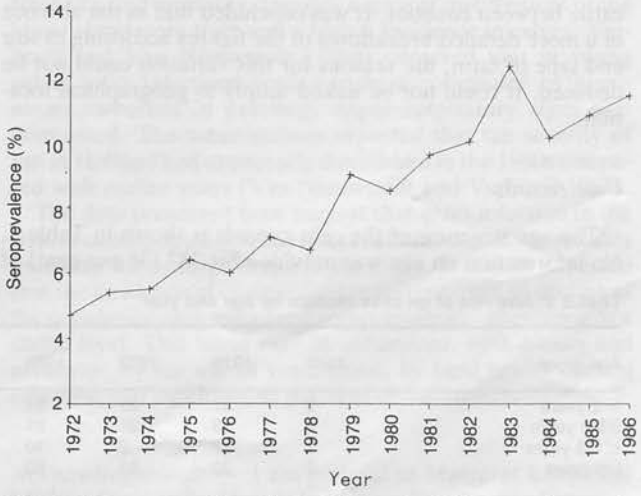

FIG 2: IBR seroprevalence in healthy cattle. (Points on the graph represent the mean of the year shown and the two preceding years)

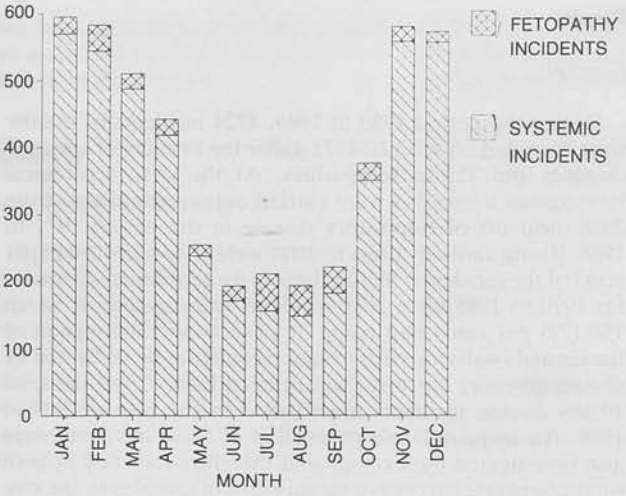

FIG 3: Monthly data for IBR/IPV on VIDA II, 1980-1986

($P<0.001$) during the study period, as illustrated in Fig 2, from below 5 per cent in the early 1970s to 10 to 12 per cent in the mid-1980s. In contrast, among samples submitted from diseased cattle (not necessarily suffering from IBR) the prevalence rose from 9·1 per cent in the period 1969 to 1973 to 34·7 per cent for 1984 to 1986 (data for the intervening years were not accessible). To estimate the prevalence of herds with serological reactors, it was calculated that 17·6 per cent of submissions from disease outbreaks tested for IBR in 1970 to 1973 had one or more seropositive animals. This figure had risen to 48·0 per cent of submissions in 1984 to 1986.

All the record systems indicated a marked seasonal trend ($P<0.001$) in IBR incidence, which is illustrated in Fig 3 using VIDA II data. Systemic (respiratory) IBR peaked in the winter period, November to February, whereas fetopathy incidents were highest in July and August.

Geographical analysis

The frequency distribution of IBR/IPV incidents by county, from the VIDA II records, was related to cattle populations recorded in the agricultural census for England (MAFF 1983), Wales (Welsh Office 1984) and Scotland (DAFS 1985). There were no consistent geographical patterns for IBR/IPV incidence (Fig 4). There was no significant correlation between the incidence rate per 100,000 cattle and either the cattle density per hectare for the county or the average number of cattle per farm for the county. The only significant correlation found was between the actual number of cases of IBR/IPV per county and the total number of cattle per county. Nevertheless, χ^2 tests indicated a significant variation in incidence per 100,000 cattle between counties. It was concluded that in the absence of a more detailed breakdown of the figures according to size and type of farm, the reasons for this variation could not be deduced. It could not be linked simply to geographical location.

Case records

The age structure of the case records is shown in Table 2. No information on age was provided for 337 (38 per cent) of

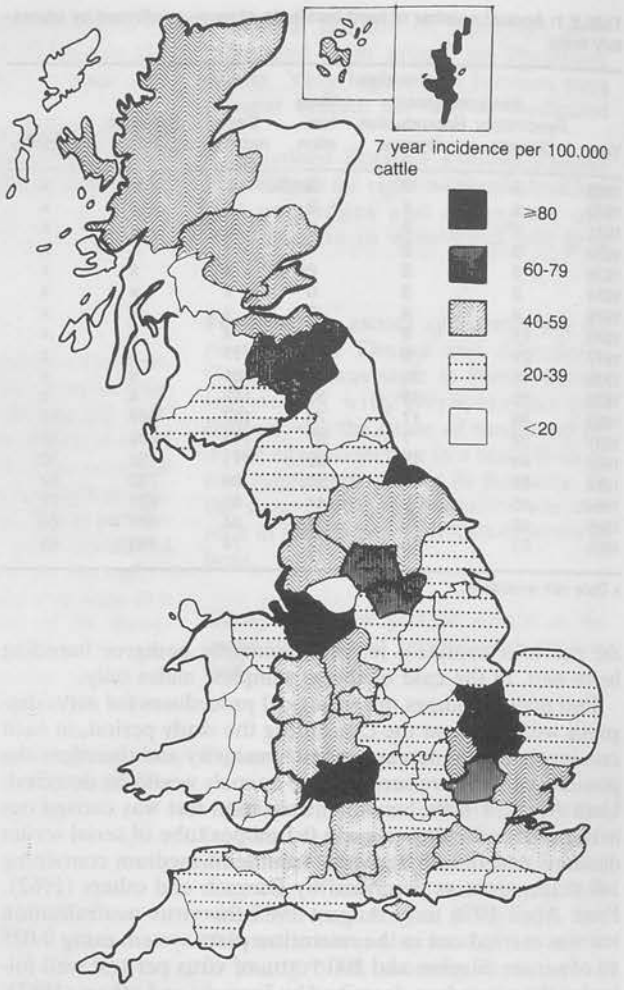

FIG 4: IBR (systemic) incidents recorded on VIDA II, 1980-1986, as proportions of county cattle populations

the incidents. The χ^2 test indicated a significant variation from the expected distribution. Notable trends in the yearly analysis were an increase in the number of calf incidents in later years and a decrease in the number of adult incidents in the early 1980s with a subsequent rise at the end of the study period.

Table 3 shows the case records distribution according to clinical signs; χ^2 was calculated for clinical signs with a total of more than 20 observations. In the yearly analysis abortion, ocular disease, pneumonia, tracheitis, mortality, and 'no history', differed significantly from the expected distribution. The proportion of cases involving abortion rose in later years. The proportion of pneumonia cases was high in the last two years of the study whereas tracheitis and mortality were both relatively more frequent in 1978 to 1979.

The data from case records were also analysed by month. Only abortion differed significantly from the general winter distribution. It was lower than predicted in the winter period (December to April) and higher from July to September.

Of the 888 cases analysed, laboratory tests used to confirm the diagnosis of BHV1 infection were: serology 460 incidents (417 by serology alone); virus isolation 460 incidents (409 by virus isolation alone); antigen detection 22 incidents (eight by

TABLE 2: Analysis of IBR case records by age and year

Age group	Year 1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	Total
> 2 years	9	31	31	26	22	15	30	25	43	40	272
0·5-2 years	4	13	21	21	18	21	15	14	13	7	147
< 0·5 years	2	7	9	10	9	14	16	18	25	22	132
Unknown	2	30	51	50	62	61	35	28	13	5	337
Total	17	81	112	107	111	111	96	85	94	74	888

TABLE 3: Analysis of IBR case records by clinical sign and year; the tabulated values are the number of incidents recorded

‡Clinical sign	Year 1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	Total	P †
Deaths	1	13	16	9	6	5	4	8	5	9	76	*
Fever	2	23	37	36	39	38	29	28	18	10	260	NS
Inappetence	2	7	11	13	14	7	5	7	6	3	75	NS
Nasal discharge/unspecified respiratory signs	14	50	61	66	62	60	45	41	45	22	466	NS
Eye discharge/ conjunctivitis	5	22	42	51	49	51	35	26	27	11	319	**
Cough	1	6	15	10	16	11	10	6	4	1	80	NS
Tracheitis	1	8	14	5	7	3	3	1	7	4	53	*
Pneumonia	1	12	21	11	12	16	10	12	28	27	150	***
Diarrhoea	2	2	8	3	2	6	4	4	1	2	34	NS
Gastrointestinal lesions		4	4	3	2	1		3	1	3	21	NS
Salivation		8	7	8	5	4	2	5	3	1	43	NS
Milk drop	3	11	12	6	8	4	11	5	5	7	72	NS
Abortion	2	6	10	11	6	5	12	11	21	23	107	***
Vaginitis	1		2				1	1	1		6	
Infertility				2	1						3	
Haemorrhagic lesions		2	2		1						5	
Skin lesions		3	1								4	
Nervous signs					2				2		4	
No history		5	14	6	8	13	16	8	3	5	78	*

† Probability that annual values differed from expected based on proportion of total records. χ^2 test NS >0·05, * <0·05, ** <0·01, *** <0·001
‡ Clinical records based on information supplied at sample submission

antigen detection alone). Antigen detection by the fluorescent antibody technique was widely used by the VI service (Edwards and others 1988) to diagnose IBR by testing nasal mucus smears, but suitable samples were only occasionally submitted to the CVL; hence the low figure in this category. The distribution of samples in which BHV1 or its antigen were detected is shown in Table 4. This indicates the predominance of the respiratory form of BHV1 infection.

TABLE 4: Summary of sample types used for BHV1 isolation and antigen detection (CVL case records); the tabulated values are the numbers of incidents in which each sample type contributed to the diagnosis

Sample	Virus isolated	Antigen detected (direct immunofluorescence)
Nasal swab	285	10
Eye swab	128	3
Lung	59	6
Trachea	42	2
Alimentary tract	18	
Blood	3	
Fetus	12	2
Vaginal swab	2	
Central nervous system	2	
Others	8	

Discussion

It is surprisingly difficult to obtain unbiased information on disease incidence. Data from submissions to diagnostic laboratories, as used here, are subject to the selection of cases by the farmer – in choosing to consult his veterinary surgeon – and by the veterinary surgeon – in deciding to submit samples to the laboratory. Such biases may not be consistent because more samples may be submitted when a disease first becomes more widely recognised, as with IBR in the late 1970s, and fewer samples may be submitted as practitioners become more confident in clinical diagnosis in the light of experience. Further confusion arises because of the changes in technical procedures used in the laboratories.

Nevertheless the data are all indicative of a sharp rise in IBR incidence in the period 1977 to 1979 although the serological results (Table 1) suggest that this rise may have begun in 1976. The data thus support both anecdotal and published reports (Anon 1979, Wiseman and others 1980) of a wave of virulent IBR at this time. It would be expected that such a dramatic increase in incidence would lead to an increasing prevalence of serological reactors in the population as a whole. This expectation is supported by the results of serology on selected healthy cattle (Fig 2), and in general diagnostic submissions where the latest figures showed 35 per cent of animals and 48 per cent of herds with evidence of prior exposure to BHV1 infection. IBR was widespread in mainland Britain and no useful geographical trends could be discerned. Although IBR showed a marked seasonality, with a peak incidence from November to February, it did occur throughout the year and the peak period for IBR-associated fetopathies was July and August, in line with the autumn calving patterns in the cattle industry.

It has been suggested that the explanation for the change in IBR incidence was the accidental importation of a virulent strain of the virus. This view is supported by molecular genetic studies of different isolates (Nettleton 1986, S. Edwards and H. White unpublished observations) and experimental calf inoculations (Msolla and others 1983, S. Edwards unpublished observations). The analysis of clinical signs in the case records needs to be approached with caution because it was based on unsolicited histories, and the absence of a record of a sign does not necessarily imply that it was absent from the particular incident. The analysis indicates the predominance of ocular and upper respiratory signs in IBR incidents. There was also evidence for a changing pattern of clinical expression during the decade 1977 to 1986 with a trend away from frank tracheitis and mortality, towards a greater involvement of the lower respiratory tract and a higher incidence in calves. Verhoeff and Van Nieuwstadt (1984) observed that in young calves BHV1 infections tended to be accompanied by pneumonia, whereas in yearlings upper respiratory signs predominated. The same authors reported that the severity of IBR in Holland had apparently diminished in the 1980s compared with earlier years (Van Nieuwstadt and Verhoeff 1983).

The data presented here suggest that BHV1 infection in the British cattle population has not yet attained enzootic stability following the upsurge in IBR in the 1970s. It may be expected that the incidence of clinical disease will continue to decline as the population immunity (and seroprevalence) rises towards a stable level. This trend may be influenced, both locally and generally, by the use of vaccination, by herd health control schemes, and by changing patterns of husbandry.

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A field trial in Belgium to control fox rabies by oral immunisation

B. Brochier, I. Thomas, A. Iokem, A. Ginter, J. Kalpers, A. Paquot, F. Costy, P.-P. Pastoret

Veterinary Record (1988) **123**, 618-621

Campaigns of fox vaccination against rabies were carried out in Belgium in September 1986 and June and September 1987. The SAD B19 attenuated strain of rabies virus was inserted into baits which were distributed over an area of 2100 km^2 at a density of 11 baits/km^2. As recommended by the World Health Organisation, the efficacy and the innocuity of the method were controlled in the field and in the laboratory. Samples of blood and brain and jaw were taken from foxes which were shot or found dead in the vaccination area, for the diagnosis of rabies, the titration of antirabies antibody and the detection of tetracycline marker. In rabid animals, the virus strain was characterised by immunofluorescence using monoclonal antibodies. In September 1987, the uptake of the baits had reached 72 per cent by 14 days after distribution. Several wild species competed with foxes in taking the baits. After the last campaign, tetracycline was found in 65 per cent of the healthy foxes collected and rabies virus neutralising antibodies were detected in 77 per cent of them. In 1987, the incidence of rabies decreased markedly in the vaccination area compared with the untreated areas. No vaccine virus was isolated either from rabid animals or from 228 small mammals trapped in the vaccination area.

SYLVATIC rabies invaded Belgium in 1966. The epizootic moved to the west and the south of the country and reached the Meuse and Sambre valleys which appear to constitute a natural barrier to the spread of the disease. The evolution of the epizootic has been characterised by a succession of peaks which, until 1982, had a mean periodicity of four years. However, after the severe outbreak in 1982, the expected lull did not occur, and in the last five years although the annual numbers of rabies cases have steadily decreased they have remained abnormally high. It may therefore be assumed that rabies persists enzootically behind the leading edge of the invasion.

Since 1967, several methods for reducing the fox population have been used unsuccessfully. As in most other European countries, these control measures were only temporarily effective and did not stop the spread of the disease. Other methods such as the oral immunisation of foxes against rabies needed to be assessed (Baer 1975). Oral administration by the

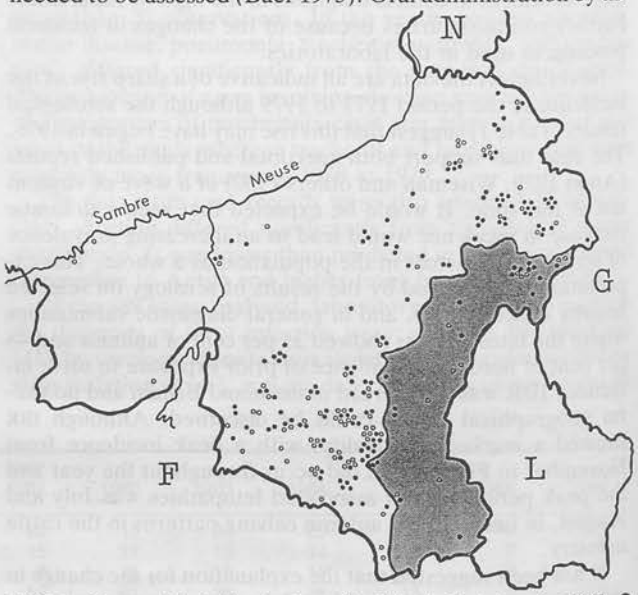

FIG 1: Geographical distribution of rabies in Belgium in 1987. ● Rabid wild animals, ○ rabid domestic animals. Dark zone indicates vaccination area

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HIGHLY SENSITIVE ANTIGEN DETECTION PROCEDURES FOR THE DIAGNOSIS OF INFECTIOUS BOVINE RHINOTRACHEITIS: AMPLIFIED ELISA AND REVERSE PASSIVE HAEMAGGLUTINATION

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ABSTRACT

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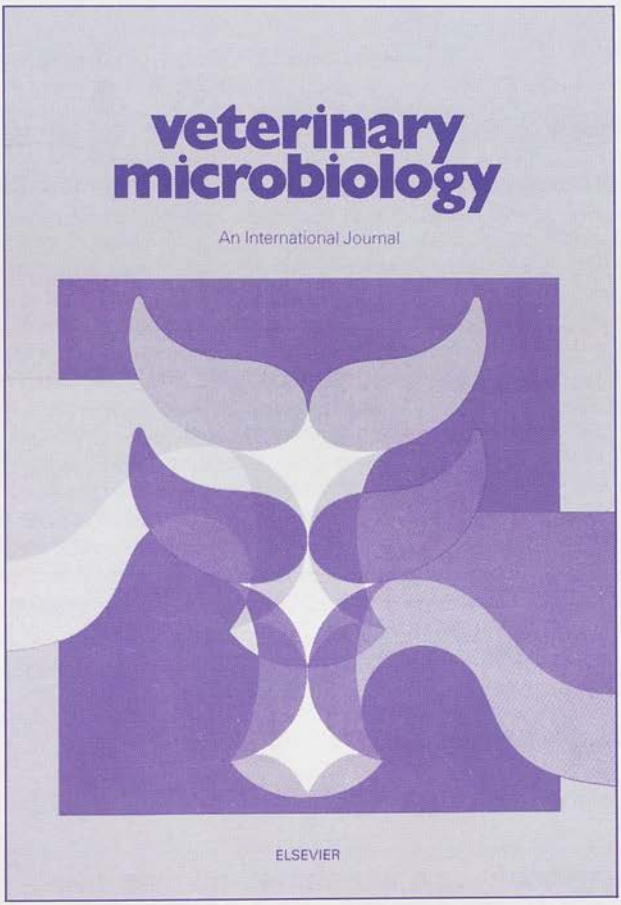

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HIGHLY SENSITIVE ANTIGEN DETECTION PROCEDURES FOR THE DIAGNOSIS OF INFECTIOUS BOVINE RHINOTRACHEITIS: AMPLIFIED ELISA AND REVERSE PASSIVE HAEMAGGLUTINATION

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ABSTRACT

Edwards, S. and Gitao, G.C., 1987. Highly sensitive antigen detection procedures for the diagnosis of infectious bovine rhinotracheitis: amplified ELISA and reverse passive haemagglutination. *Vet. Microbiol.*, 13: 135—141.

The sensitivity of an enzyme-linked immunosorbent assay (ELISA) for the detection of bovid herpesvirus 1 antigen was increased by up to 50-fold using the biotin-avidin interaction to amplify the reaction, when compared with a simple sandwich ELISA. An alternative immunoassay, reverse passive haemagglutination (RPHA), had a similar sensitivity to the amplified ELISA, and was technically simpler to perform. Both the amplified ELISA and the RPHA could detect viral antigen in the nasal secretions of calves undergoing experimental primary infection with the virus from Day 3 to Day 7 after inoculation. Neither assay was as sensitive as virus isolation in cell culture and they failed to detect antigen in virus-positive samples from the calves from 8 days after inoculation, and from vaccinated calves undergoing challenge infection.

INTRODUCTION

The specific diagnosis of infectious bovine rhinotracheitis (IBR) has traditionally been based on the isolation of the causal virus (bovid herpesvirus 1; BHV1) in cell culture, together with the detection of an active antibody response in the host animal (Gibbs and Rweyemamu, 1977). The detection of viral antigen in clinical samples can be a rapid and economical alternative to cell culture. The most widely applied antigen detection technique has been immunofluorescence, either on smears of cells from the nasal or ocular epithelium (Terpstra, 1979; Nettleton et al., 1983; Silim and Elazhary, 1983) or on cryostat sections of tissues collected at post mortem (Terpstra, 1979). Immunoenzymatic techniques have been described for the labelling of infected cells (Edwards et al., 1983) and for

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the detection of soluble antigen in diluted nasal mucus (Faye et al., 1979; Nettleton et al., 1982; Collins et al., 1985).

Edwards et al. (1983) found that none of these three antigen detection methods for BHV1 attained the sensitivity of virus isolation in cell culture. Guesdon et al. (1979) developed enzyme immunoassays of high sensitivity by exploiting the biotin-avidin interaction; the present study describes the application of their techniques in an enzyme-linked immunosorbent assay (ELISA) for BHV1 antigen and compares it with an alternative employing reverse passive haemagglutination (RPHA).

MATERIALS AND METHODS

Samples

The assays were optimised, and their comparative sensitivities assessed, using a simple freeze-thaw harvest of BHV1 grown in MDBK cell cultures, and with virus from the same harvest purified on a 20—50% potassium tartrate gradient centrifuged at 80 000 × *g* for 1 h.

Twenty four calves were experimentally infected with 10^7 $TCID_{50}$ of a field strain of BHV1 by intranasal inoculation either as a primary infection or following vaccination against the virus (Edwards et al., 1986). Cotton wool nasal swabs were taken daily from 2 to 10 days after the inoculation, and eluted into 3 ml phosphate buffered saline with 0.5% lactalbumen hydrolysate and antibiotics. This constituted a dilution factor of approximately 1/20 of the nasal mucus. The samples were stored at −70°C to await testing. After clarification by low speed centrifugation the supernatant of this extraction medium was tested by the antigen detection assays without further treatment or dilution.

ELISA

The viral antigen concentration in the sample extraction medium was measured by ELISA following three protocols. The simple sandwich (SS) method was performed as described by Edwards et al. (1983) using gnotobiotic calf antibody to the virus conjugated directly with peroxidase as the detector system. The avidin-biotin ELISAs were developed from those of Guesdon et al. (1979). In the labelled avidin-biotin (LAB) system a two-stage detection system was used, namely biotinylated antiviral antiserum with a 2:1 molar ratio of biotin:immunoglobulin, followed after washing by an avidin-peroxidase conjugate (Sigma type VI) at a dilution of 1/1000. The bridged avidin-biotin (BAB) system employed biotinylated antiviral antibody at a 4:1 molar ratio of biotin:immunoglobulin, followed after washing by avidin (Sigma) 10 $\mu g\ ml^{-1}$ then, after further washes, biotinylated horseradish peroxidase (molar ratio 10:1 biotin:peroxidase). For all three ELISA techniques the initial coating of microtitre plates with antiviral

antibody, the addition of test samples containing possible viral antigen, and the final colour development using *o*-phenylene diamine as chromogen, were as described previously (Edwards et al., 1983).

The biotinylation of the proteins for the LAB and BAB assays was carried out by adding the appropriate quantities of 0.1 M biotin-N-hydroxysuccinimide dissolved in dimethylformamide to a 10 mg ml^{-1} solution of the protein in 0.1 M sodium bicarbonate. After reacting for 1 h at room temperature, the unbound biotin was removed by dialysis against phosphate buffered saline pH 7.2. For the optimisation of the assays the most suitable degree of biotinylation of each protein and the most appropriate concentration of each reagent were determined by a series of two-dimensional chequerboard titrations using known positive and negative virus samples. Each step of the ELISA procedures was incubated for 1 h at 37°C in a moist chamber, apart from the substrate which was incubated for 15 min at room temperature.

Controls for each sample in all the ELISAs consisted of parallel tests in wells coated with antibody negative to BHV1. To calculate the final test results, the optical density value in the control well for each sample was subtracted from the value in the test well, thus compensating for variation in the non-specific colour reaction obtained with different samples.

RPHA

Sheep red blood cells were washed, then treated with chymotrypsin as described by Cranage et al. (1985). Anti-viral specific immunoglobulin was prepared from gnotobiotic calf antiserum to BHV1 using the caprylic acid method (Steinbuch and Audran, 1969). After dialysis against 0.9% sodium chloride the antibody was coupled to the enzyme-treated erythrocytes using chromic chloride (Scott et al., 1981). A 1% suspension of the coated cells was stabilised by glutaraldehyde treatment (Cranage et al., 1983a) and kept at 4°C. The test was carried out by mixing 30 μl of serial doubling dilutions of swab extraction media with equal volumes of 1% coated red cell suspension in a 96-well round-bottomed microtitration plate. After 2 h at room temperature the haemagglutination reaction in each well was graded visually on a scale from 0 (absent) to 4 (complete agglutination). The end point was taken as the highest dilution giving a reading of $\geqslant 2$. The controls used for each sample were uncoated sheep erythrocytes, and sheep erythrocytes coated with antibody negative to BHV1.

RESULTS

The SS ELISA could detect gradient-purified virus at a concentration equivalent to $10^{6.0}$ $TCID_{50}$ ml^{-1}, whereas the amplified ELISAs could detect considerably smaller amounts of the same preparation ($10^{4.5}$ $TCID_{50}$ ml^{-1} and $10^{4.2}$ $TCID_{50}$ ml^{-1} for the LAB and BAB system respectively). This

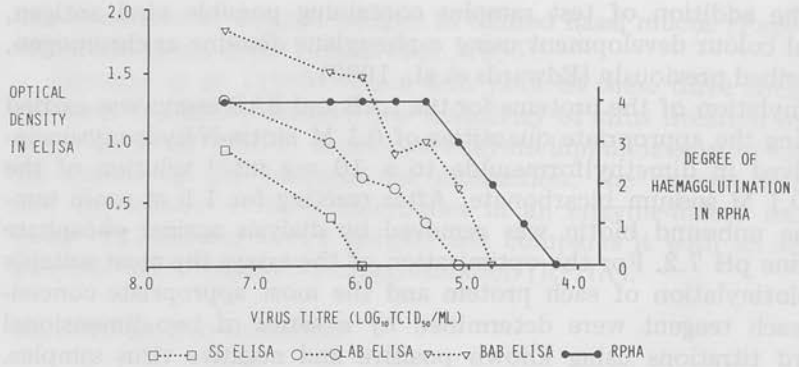

Fig. 1. Comparative sensitivities of three enzyme-linked immunosorbent assay techniques and reverse passive haemagglutination for the detection of bovid herpesvirus 1 in cell culture fluids.

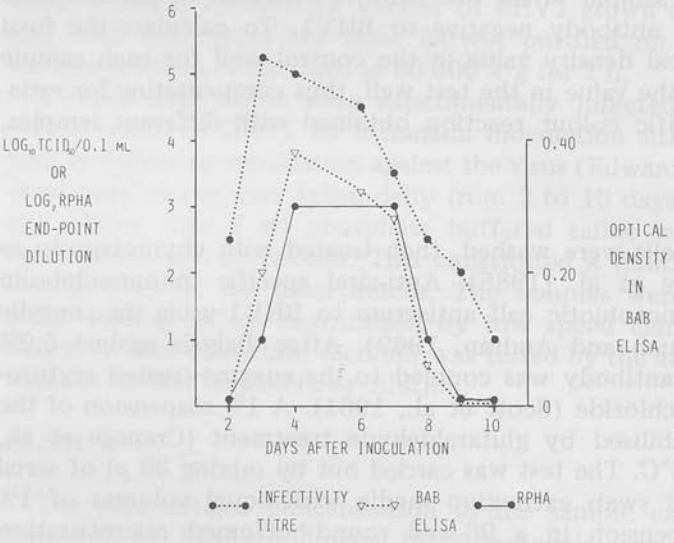

Fig. 2. Virus infectivity, and antigen detected by amplified enzyme-linked immunosorbent assay and reverse passive haemagglutination, in the nasal secretions of a single calf following intranasal inoculation of 10^7 $TCID_{50}$ bovid herpesvirus 1.

was equivalent to a detection rate for BAB of 5 ng viral protein per test well (100 ng ml^{-1}). The sensitivity of the assays was somewhat lower for the crude cell culture harvest of the virus, although the enhanced sensitivity of the amplified systems was still apparent. The sensitivities of the four antigen detection systems are compared in Fig. 1, using the unpurified virus preparation. RPHA had a detection limit similar to those of the amplified ELISAs.

The most sensitive of the ELISAs (BAB) was compared with RPHA

for the detection of BHV1 antigen in the experimental calf samples. A positive result was achieved with only one sample from the vaccinated calves undergoing challenge infection. This sample (taken on Day 4 after challenge) had an infectivity titre of $10^{6.0}$ $TCID_{50}$ ml^{-1}. Of the other 79 vaccinated calf samples, 40 contained infectious virus detectable in cell culture and all had titres of $\leqslant 10^{5.5}$ $TCID_{50}$ ml^{-1}.

In the unvaccinated calves undergoing primary challenge with BHV1, both antigen detection tests gave positive results for samples with a wide range of infectivity titres ($10^{3.5}$—$10^{6.75}$ $TCID_{50}$ ml^{-1}). The two antigen detection tests showed a high degree of agreement (r = 0.84) but the correlation between antigen level and infectivity titre was much lower. Figures 2 and 3 illustrate the pattern of virus and antigen shedding in the nasal secretions of a representative single calf (Fig. 2) and the mean values for the group (Fig. 3). The infectivity titre of virus appeared to rise faster than the detectable antigen level in the first 3 days after inoculation. Thereafter the curves for the antigen detection tests follow that for the infectivity titre except that infectious virus remained detectable at a moderate to low level ($\leqslant 10^{5.25}$ $TCID_{50}$ ml^{-1}) for several days when the antigen detection tests had become negative.

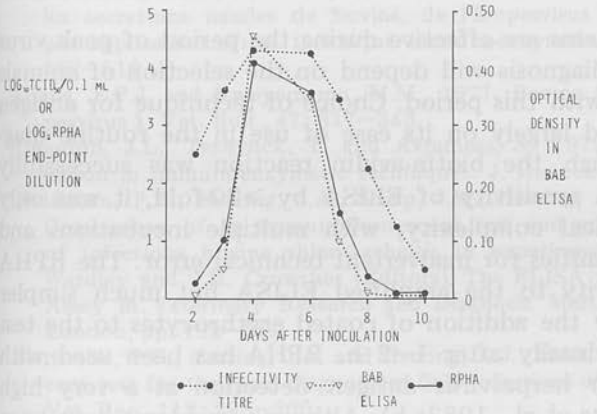

Fig. 3. Group mean infectivity titres, and antigen level detected by enzyme-linked immunosorbent assay and reverse passive haemagglutination, in the nasal secretion of 13 calves following intranasal inoculation of 10^7 $TCID_{50}$ bovid herpesvirus 1.

DISCUSSION

BHV1 survives well in clinical samples, and is readily isolated in cell culture. No antigen detection technique has yet been described which can rival the sensitivity of isolation in cell culture as a method for identifying this virus in a sample. Collins et al. (1985), using a carefully optimised SS ELISA utilising monoclonal antibodies, could detect $10^{5.5}$ $TCID_{50}$ ml^{-1} of BHV1 in nasal swab samples prepared as in the present study. This

is similar to the sensitivity of the amplified ELISA and the RPHA presented here. Some caution should be exercised in assessing the sensitivity of antigen detection techniques in terms of the infectivity titres of virus. The results suggest that the amount of detectable antigen is not directly related to infectivity, and that in the early days after infection the calves' nasal secretions contain a higher proportion of infectious virus to total antigen than from Day 4 onwards. A similar trend may be observed in the results presented by Collins et al. (1985). Antigen detection methods ceased to be useful diagnostic tools from 8 days after the initial infection. This has also been reported by other authors using a variety of techniques (Terpstra, 1979; Edwards et al., 1983; Collins et al., 1985) although the height and duration of positive reactions in antigen detection tests varies with the breed and age of calf and the dose and route of virus inoculum (Edwards, unpublished observations).

The infectivity curves of nasal secretions from individual calves (Fig. 2) showed a dramatic drop of 100- to 1000-fold in virus titre over a 2-day period from Day 7 to Day 9 after inoculation. This presumably coincides with the onset of the immune response. For antigen detection systems to be effective beyond this period, or at any stage in immune animals experiencing reinfection (e.g. following vaccination), a major increase in assay sensitivity will be required.

All of the available systems are effective during the period of peak virus shedding, and success in diagnosis will depend on the selection of animals for sampling to coincide with this period. Choice of technique for antigen detection will then depend largely on its ease of use in the routine diagnostic laboratory. Although the biotin-avidin reaction was successfully used here to increase the sensitivity of ELISA by $\geqslant$20-fold, it was only at the expense of technical complexity, with multiple incubations and washes, and many opportunities for inadvertent technical error. The RPHA was equivalent in sensitivity to the amplified ELISA but much simpler to conduct, involving only the addition of coated erythrocytes to the test solution, and reading it visually after 1—2 h. RPHA has been used with monoclonal antibodies for herpesvirus antigen detection at a very high level of sensitivity (Cranage et al., 1983a,b). Although problems have been reported with RPHA on samples of nasal secretion, due to non-specific haemagglutination (Cranage et al., 1981), this did not arise in the present study. This will require further study on samples from cattle with naturally occurring respiratory disease to assess its likely importance.

Despite the limitations of sensitivity, antigen detection techniques are being increasingly used in the diagnosis of IBR, with justification on the dual grounds of speed and economy. Two techniques described here (amplified BAB ELISA and RPHA) have a sensitivity which is possibly as high as can be achieved with this type of technology, can be performed, if necessary, with simple inexpensive laboratory equipment, and are more objective in test evaluation than, for example, immunofluorescence.

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Research in Veterinary Science 1983, 34, 42–45

Experimental infectious bovine rhinotracheitis: comparison of four antigen detection methods

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The duration of detectable viral antigen in nasal secretions was studied by four methods in calves experimentally infected with infectious bovine rhinotracheitis. Virus isolation in cell culture was the most sensitive and was successful for up to 11 days after inoculation. Three direct rapid antigen detection systems (immunofluorescence, immunoperoxidase and enzyme-linked immunosorbent assay [ELISA]) were all successful during the pyrexic phase, but less useful in the later stages of the disease. Virus isolation and ELISA remained positive in strongly positive samples stored for a week at room temperature.

ALTHOUGH bovid herpesvirus 1 (BHV 1) is readily isolated in cell culture from most cases of infectious bovine rhinotracheitis (IBR) many laboratories lack culture facilities and there is a need for alternative and more rapid diagnostic methods. This paper presents the results of a comparison between three rapid antigen detection techniques and virus isolation in cell culture, in nasal swab samples from experimentally infected calves.

Materials and methods

A recent (1980) field isolate of BHV 1 was grown in primary calf kidney cell culture, harvested by one freeze-thaw cycle and stored at —70°C. Three six- to seven-month-old Friesian calves were inoculated intranasally with 4×10^8 TCID50 of the virus at the fourth cell passage. Three control calves in an adjacent but separate pen were inoculated with uninfected cell culture harvest. All animals were examined daily including rectal temperature measurement for seven days before and 14 days after inoculation.

Cotton wool nasal swab samples were taken daily and immediately broken off into 3 ml sterile phosphate buffered saline (PBS) pH 7·2 in small screw capped bottles. These samples were agitated on a vortex mixer and the swab removed. The nasal secretion suspension was then centrifuged at 500 *g* for five minutes. The supernatant was reserved for virus isolation and enzyme-linked immunosorbent assay (ELISA). The preparation of cell smears was based on the methods of Minnich and Ray (1980) and Thomas and Stott (1981). The cell deposit was mixed with 1 ml PBS and single drops were spotted onto PTFE coated multispot glass slides (C. A. Hendley) and evaporated to dryness either rapidly (45 minutes) at 37°C or overnight at 4°C. The slides were fixed in acetone for 10 minutes and rinsed in distilled water before labelling by direct immunofluorescence or immunoperoxidase techniques.

The IgG fraction of a gnotobiotic bovine antiserum to BHV 1 was prepared by the method of Reif (1969). Immunoglobulin was conjugated with fluorescein isothiocyanate by a method based on that of Nairn (1976). Horseradish peroxidase (Sigma type VI) conjugate was prepared by the sodium periodate method of Wilson and Nakane (1978) but was used without Sephacryl purification as this had been found unnecessary (Chasey 1980). Both conjugates were stored as stock preparations at —20°C after dilution with an equal volume of glycerol.

Smears for immunofluorescence were incubated with a one in four dilution of stock conjugate in PBS for 30 minutes at 37°C, washed for 10 minutes in each of three changes of PBS and counterstained with Evans blue diluted 1 in 100,000 for one minute. They were mounted in 50 per cent buffered glycerol for microscopic examination under incident ultraviolet light.

Smears for immunoperoxidase labelling were reacted with stock conjugate, diluted one in five, for 30 minutes at room temperature. After rinsing in 0·1 M phosphate buffer, pH 7·2, they were covered with a solution of 3 3′ diaminobenzidine (DAB) for 10 minutes (Chasey 1980), and washed in tap water before being lightly counterstained with toluidine blue. The smears after rinsing and drying were mounted in DPX mountant and examined in bright field. Duplicate smears were processed similarly but incubated with 'inappropriate' conjugates specific

for heterologous virus (bovine virus, diarrhoea virus or respiratory syncytial virus), and assessment of specific IBR labelling was made by comparison.

The ELISA was performed in microtitre plates (M129A; Dynatech) which were coated overnight at 4°C with 100 µl per well of anti-BHV 1 IgG preparation, 4 µg/ml in 50 mM carbonate buffer pH 9·6. The plates were washed three times in 0·05 per cent Tween 20; 100 µl aliquots of test sample supernatants were added in duplicate to the wells and incubated for two hours at 37°C. After three more washes, 100 µl of anti-BHV 1 peroxidase conjugate was added at a dilution from stock of 1 in 1000. The conjugate diluent was 0·5 M sodium chloride buffered to pH 7·2 with phosphate, containing 0·05 per cent Tween, 1 mM EDTA, 4 per cent polyethylene glycol 6000 and 1 per cent bovine albumen. The plates were incubated for one hour at room temperature, washed three more times in 0·05 per cent Tween and to each well was added 200 µl ortho phenylene diamine 0·4 mg/ml in 0·075 M phosphate citrate buffer pH 5·0 with 0·01 per cent hydrogen peroxide. The colour reaction was stopped after 20 minutes at room temperature by the addition of 50 µl per well 2·5 M sulphuric acid. Optical density (OD) was measured at 492 nm on a Titertek Multiskan photometer.

For virus isolation, calf kidney secondary cell cultures were seeded into microtitre plates with Eagle's minimum essential medium plus 10 per cent fetal calf serum and incubated at 37°C in 5 per cent carbon dioxide atmosphere until confluent monolayers formed. Ten µl of sample supernatants were added in duplicate to wells containing 70 µl of medium. The cells were examined daily for four days for signs of cytopathic effect. The final identification of virus growth was by immunoperoxidase labelling of the cells in the microplates. The medium was carefully decanted and the cells fixed with 2 per cent formaldehyde in PBS for 15 minutes at 4°C. The plates were rinsed in PBS for five minutes, then 15 minutes. Twenty-five µl per well of anti-BHV 1 peroxidase conjugate was added, diluted 1 in 50 in 0·5 M sodium chloride plus 1 per cent Tween 80, buffered with phosphate to pH 7·6. Plates were incubated for 15 minutes at 37°C then rinsed with PBS containing 1 per cent Tween 80 for five minutes, then 15 minutes; 25 µl per well of DAB substrate was added for 20 minutes at room temperature, after which the wells were emptied and 100 µl PBS added for reading under an inverted microscope.

Preliminary trials had established the optimal concentrations of reagents used in all the immunoassays, and checked the specificity of the reactions.

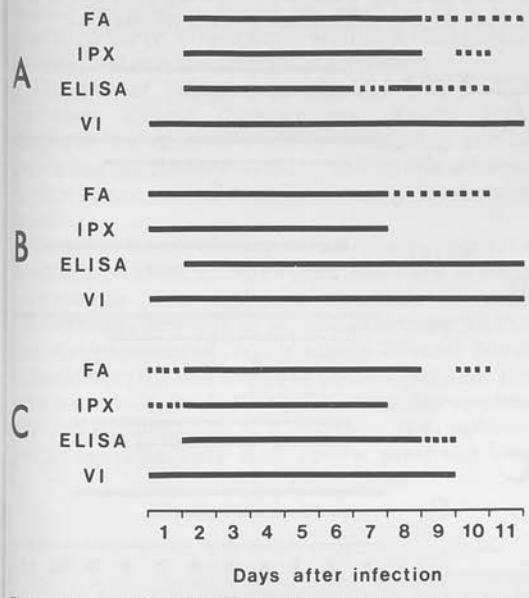


FIG 1: Duration of BHV 1 detectability for infected animals A, B and C, by four techniques; direct immunofluorescence of nasal secretion cells (FA), direct immunoperoxidase on nasal secretion cells (IPX), enzyme linked immunoassay for soluble antigen in nasal secretion (ELISA) and virus isolation with confirmation by immunoperoxidase (VI). Solid bars represent positive results, broken bars weak positive

Results

Clinical response

The three infected animals showed a significant rise in rectal temperature above pre-inoculation values from the second to sixth (animal A) or seventh days (animals B and C). Clinical signs of IBR (nasal and ocular discharges and conjunctivitis) appeared on the second (animal C) or fourth day (animals A and B) and persisted in all animals until the 11th day with a slight nasal discharge still present in animal B and a mild ocular discharge in animal C up to the 14th day. Control animals had a normal rectal temperature and showed no clinical signs throughout the test period.

Virus detection

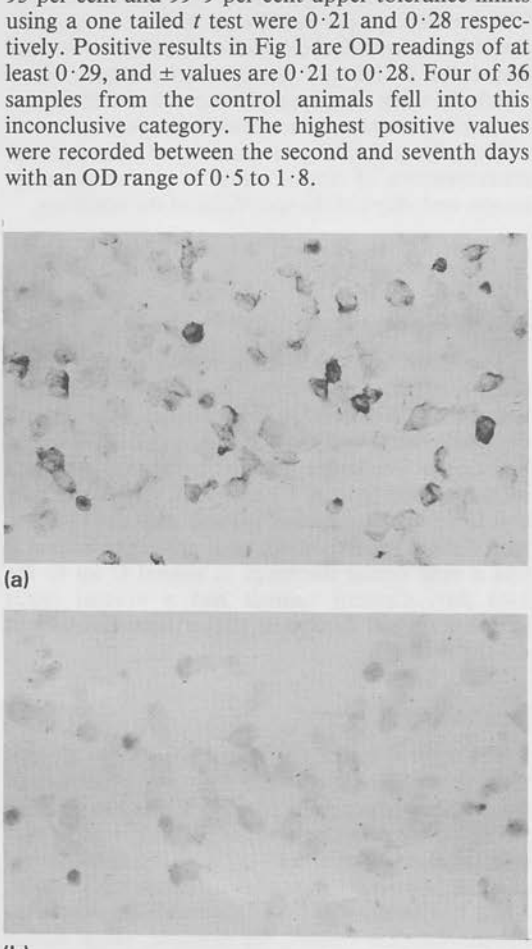
The control animals remained negative to all tests throughout the experiment, with the exception of four inconclusive results to ELISA (see below). The results of the four test systems on nasal swabs from infected animals are shown in Fig 1. Readings classed as weak positive indicate a result which, taken alone, could not be used to establish a definite diagnosis. Immunolabelled smears, for example, taken during the later stages of infection often contained only one or two positive cells.

The proportion of positive cells in the smears was greatest from the second to the sixth days after

inoculation (all over 10 per cent, reaching a peak of over 50 per cent on the third day). From the seventh day this proportion was markedly reduced (1 to 4 per cent positive cells) and fell to below 1 per cent in most samples thereafter, but even a level of 1 per cent positive cells was easily detected especially in fluorescent labelled smears when non-specific background fluorescence was so low as to give a strong contrast with specifically labelled cells.

With immunoperoxidase the interpretation was greatly aided by a comparison of the specifically labelled and control smears (Fig 2). Endogenous peroxidase activity was insignificant.

For the ELISA test the negative baseline level was determined from the readings of the control animal samples. The mean OD_{492} for these was 0·13 and the 95 per cent and 99·9 per cent upper tolerance limits using a one tailed *t* test were 0·21 and 0·28 respectively. Positive results in Fig 1 are OD readings of at least 0·29, and ± values are 0·21 to 0·28. Four of 36 samples from the control animals fell into this inconclusive category. The highest positive values were recorded between the second and seventh days with an OD range of 0·5 to 1·8.



(a)

(b)

FIG 2: Duplicate nasal smears, lightly counterstained, from an infected animal three days after infection, labelled directly with immunoperoxidase conjugates specific for BHV 1 (a) and a heterologous virus (b). ×200

The ELISA colour reactions could also be read by eye and there was no discrepancy between visual and photometer results for positive or negative samples. Most of the inconclusive OD values were interpreted visually as negative.

Effects of room temperature storage

The samples were stored for up to seven days at room temperature as either washed cell suspensions or as sample supernatants. The effect on cells was very variable and, although some samples could still be read with confidence by immunofluorescence or immunoperoxidase methods after seven days storage, the majority were only satisfactory up to three days. After this there was a progressive disruption of cell architecture and a marked reduction in the number of intact cells in the smears. The actual labelling, particularly with fluorescent antibody, remained strong indicating persistence of the viral antigen, but its interpretation became difficult when it could not be related to intact cells.

The effect of seven day storage on ELISA and virus isolation results is shown diagrammatically in Fig 3. The strong positives remained positive but there was a reduction in positive results for both tests from the eighth day after inoculation. Gross bacterial or fungal contamination of some samples was evident after the week at room temperature, but this did not appear to interfere with the results.

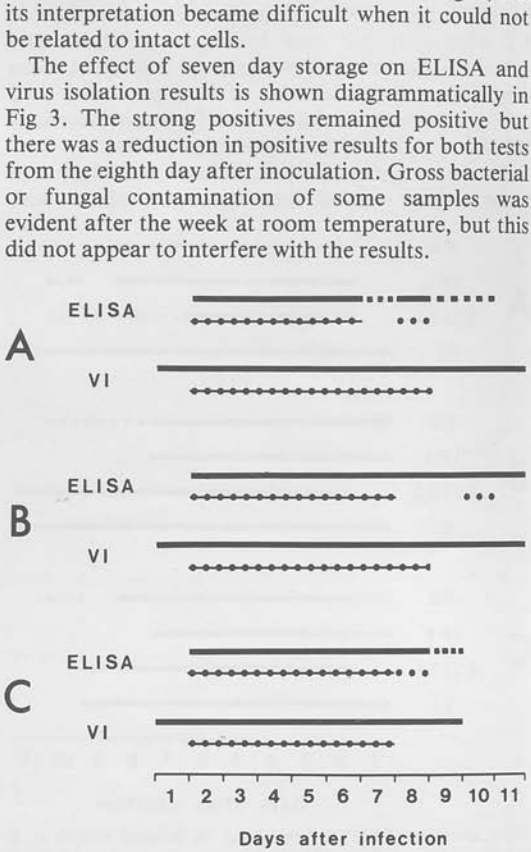


FIG 3: Effect of room temperature storage on ELISA and virus isolation (VI) results for infected animals A, B and C. Continuous bars represent positive results and broken bars weak positive. ——— Test on sample date, ●—●—●—● Test after one week at room temperature

Discussion

All four tests for virus or viral antigen were positive from the second to sixth or seventh days after inoculation. This coincided with the duration of pyrexia in the individual animals. On the first day and after the seventh day the most sensitive assay was virus isolation, despite the fact that the technique employed was designed for ease of application rather than maximum sensitivity. Conventional tube cultures with repeated passages may well have detected virus in the nasal secretions for longer periods. The results for immunofluorescence and virus isolation are in agreement with those of Terpstra (1979).

None of the three antigen detection systems stood out as markedly better than the other two, and any or all of them could be developed as diagnostic tests by laboratories lacking cell culture facilities. Provided a number of animals were sampled in the febrile phase of disease, a diagnosis of an outbreak should be possible by these means. All the tests can be completed within a day of receiving a sample. The success of these immunoassays was considered to be dependent on the quality of the detector antiserum, and previous attempts with non-gnotobiotic sera had been less encouraging. The preparation method for cell smears was also important in order to obtain sufficient cells for labelling. Simple nasal mucus smears prepared by wiping the swab tip on a slide produced 'dirty' preparations with high background staining and very few cells for examination.

The use of polyethylene glycol in the ELISA conjugate diluent (Salonen and Vaheri 1981) increased the ratio of positive to negative OD by increasing the positive readings, and bovine albumen further improved the ratio by reducing the negative values.

Delays in transit of samples to the laboratory are a common problem and even after one week at room temperature virus isolation remained the most sensitive test, although its advantage over the ELISA was less pronounced. Again acutely infected febrile animals were the best source of positive material. The relative stability of BHV 1 (Gibbs and Rweyemamu 1977) is advantageous in overcoming this problem. Nasal secretion cells were poorly preserved with prolonged room temperature storage in aqueous suspension, and if the fluorescent antibody or immunoperoxidase techniques are to be used it is better to prepare and fix the smears as close to the point of origin as possible. Fixed smears can be sent without problem through normal mail services.

The decision as to which test to use for IBR diagnosis will depend on facilities available. Virus isolation is to be preferred if possible, although one of the rapid antigen detection systems used in parallel may enable earlier results to be produced. Provided the appropriate antiserum and reagents are available, the ELISA requires the least specialised equipment if read by eye. Immunofluorescent labelling needs an ultraviolet microscope, while immunoperoxidase techniques require only simple bright field optics and, in addition, produce permanent preparations. Slides prepared by either method are easily interpreted by an experienced worker.

The immunoperoxidase and ELISA results were very encouraging although both techniques need to be fully proven on clinical samples from field cases of IBR.

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A Veterinary Service Scheme for the Rapid Diagnosis of Viral Infections in Ruminants, using Immunofluorescence

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Summary

The immunofluorescence technique was developed at the Central Veterinary Laboratory for use as a rapid test for viruses in tissue sections and smears from cases of respiratory disease, mucosal disease and abortion in cattle, and border disease in sheep. Once reliably established the technique was transferred to Veterinary Investigation Centres by means of a training programme and the supply of equipment, conjugates and quality control materials. Results obtained in the scheme have demonstrated the importance of respiratory syncytial virus and infectious bovine rhinotracheitis virus in bovine respiratory disease. Marked seasonal trends for these two infections have also been revealed.

Introduction

Although applications of immunofluorescence (IF) for the diagnosis of viral infections were being reported 30 years ago. Emmons and Riggs commented in 1977 that routine use of IF was still surprisingly rare. Among the reasons they suggested were non-availability of reliable reagents, inadequate quality control, lack of experienced laboratory personnel and lack of confidence in the technique among clinicians. This article describes attempts by the Ministry of Agriculture, Fisheries and Food (MAFF) veterinary service to address the first three problems in the hope of thereby solving the fourth. Particular emphasis was given to the application of the IF technique for diagnosis of bovine respiratory diseases.

At the Central Veterinary Laboratory (CVL) IF has been used for many years in the diagnosis of viral infections such as rabies and swine fever. The emergence of infectious bovine rhinotracheitis (IBR) as a major disease problem in the late 1970s (Wiseman and others, 1978) led to a demand for more specific diagnostic methods, much of which has been met by the IF technique (Nettleton and others, 1983).

A valuable feature of the IF technique is its applicability to agents, such as bovine respiratory syncytial virus (RSV) (Thomas and Stott, 1981), which are difficult to diagnose by conventional cell culture techniques. A further advantage of IF is its rapidity, with test reports being possible within hours of receipt of samples at the laboratory. To achieve this, however, it was considered that local testing at Veterinary

Investigation Centres (VICs) would be the ideal so as to avoid the inevitable delays in shipment to, and reporting from, the specialist central laboratory.

Materials and methods

For the scheme the direct IF method was used for viral antigen detection. Specific antisera were prepared by immunising colostrum-deprived calves with live virus by the intranasal route, with one or more intranasal booster inoculations being given at four to six week intervals, and a final intravenous boost seven days before slaughter and bleeding out. Batches of serum were conjugated to fluorescein isothiocyanate (FITC) following the method of Nairn (1976); the conjugates were then lyophilised in 2ml aliquots and stored at 4°C in the dark. Frozen sections of tissue (5μm), impression smears of lung, and dried smears of washed nasal epithelial cells were fixed in acetone, stained with conjugate at a predetermined dilution in phosphate buffered saline pH 7.6 (PBS) for 30 minutes at 37°C, washed in PBS for 30 minutes, and counterstained with Evans' blue. They were then examined by epifluorescent microscopy using a ×40 objective lens. Samples of positive (and negative) control material, prepared from cell cultures which had been infected with the respective viruses, were used for comparison.

In order to evaluate the method and the reagents, commencing in 1982 all bovine lung tissue submitted from VICs to the CVL for virological examination was tested by IF for the presence of IBR, RSV, parainfluenza 3 (PI3) and bovine virus diarrhoea (BVD) viral antigens. Subsequently the range was extended to include bovine adenovirus (subgroup A). In addition, tissues from cases of suspected BVD-mucosal disease and ovine border disease were tested for BVD antigen. From bovine abortions, fetal tissues were tested for BVD and IBR antigens, and fetal fluids by indirect IF for BVD and IBR antibodies.

The subsequent programme included four main components: (a) training courses held at the CVL at which selected staff from all MAFF VICs were given instruction in the procedures for IF diagnosis and in the recognition of specific fluorescence patterns exhibited by the different viruses; (b) allocation of the necessary resources to VICs to up-date their equipment; (c) the preparation at CVL and supply to VICs of the specific fluorescent conjugates, and also the positive and negative materials for quality control purposes; (d) incorporation of virological IF into the MAFF quality assessment scheme in which identical samples are sent to all participating laboratories to test and report (Johnson, 1988*). A summary of tests carried out, and positive diagnoses, was sent monthly by VICs to the CVL, where the data were stored and analysed on computer. Some results from use of IF diagnosis at VICs were available in 1984 but the scheme was not fully operational until the following year.

Results

Results of IF tests done at the CVL are summarised in Table 1. A comparison of this antigen detection method with virus isolation in cell culture for lung samples during the first two years has been reported previously (Edwards and others, 1984). The success of the IF method, particularly for the diagnosis of RSV, led to the decision to decentralise viral diagnosis wherever possible and this transfer of work from CVL to VICs accounts for the fall in number of tests done at CVL in 1986.

*P.36

Table 1. Results of immunofluorescent tests for viral diagnosis on ruminant samples at the CVL, 1982–1986

Year	Bovine Respiratory Disease				Bovine abortion				BVD/ mucosal disease		Border disease	
	Lungs		Nasal smears		Tissues		Fluids					
	a	b	a	b	a	b	a	c	a	b	a	b
1982	293	2(I) 11(R) 1(B)	151	18(I)	90	1(B)	46	1(B)	240	12(B)	46	4(B)
1983	393	1(I) 8(P) 33(R)	164	6(I) 1(R) 1(B)	226	1(I) 1(B)	206	6(B)	246	16(B)	70	5(B)
1984	359	2(I) 12(P) 39(R)	114	3(I) 2(P)	356	1(B)	284	13(B)	269	33(B)	88	7(B)
1985	311	1(I) 5(P) 34(R) 1(B)	66	4(I) 2(R) 1(P)	–	–	285	15(B)	209	20(B)	3	–
1986	143	2(I) 4(R) 1(B)	33	–	–	–	261	17(B)	46	5(B)	49	6(B)

a = No. cases (animals) tested; b = no. positive for antigen; c = no. positive for antibody
I = IBR; P = PI3; R = RSV; B = BVD–BD

Table 2 gives a summary of VIC returns for the two year period 1985–1986. The first two columns indicate the diagnostic rate on an outbreak basis. The remaining columns indicate the success rate for individual live animals (e.g. nasal mucus smears) and for individual animals at post-mortem (smears and frozen sections of tissues). Undue emphasis should not be given to the percentage diagnosis rates for categories where the total number of investigations was low.

As shown in Fig. 1, there was a marked seasonality for IBR and RSV diagnoses with the highest occurrence of both diseases from November to February. Many fewer investigations for respiratory disease were done in the summer but, even so, the proportion positive for IBR and RSV was lower. For example, of the 1 409 IBR investigations in the January to March periods, 237 (17 per cent) were diagnosed as positive compared with only 38 (7 per cent) of the 539 investigations from June to August. Comparative figures for RSV were 187/1 417 (13 per cent) from November to January and 9/373 (2 per cent) from June to August. The seasonal pattern was less marked for PI3 and absent for BVD. No significant geographical differences could be discerned when the data were analysed by VIC of origin.

Table 2. Virus diagnosis by immuofluorescence reported by Veterinary Investigation Centres in England and Wales, 1985–86

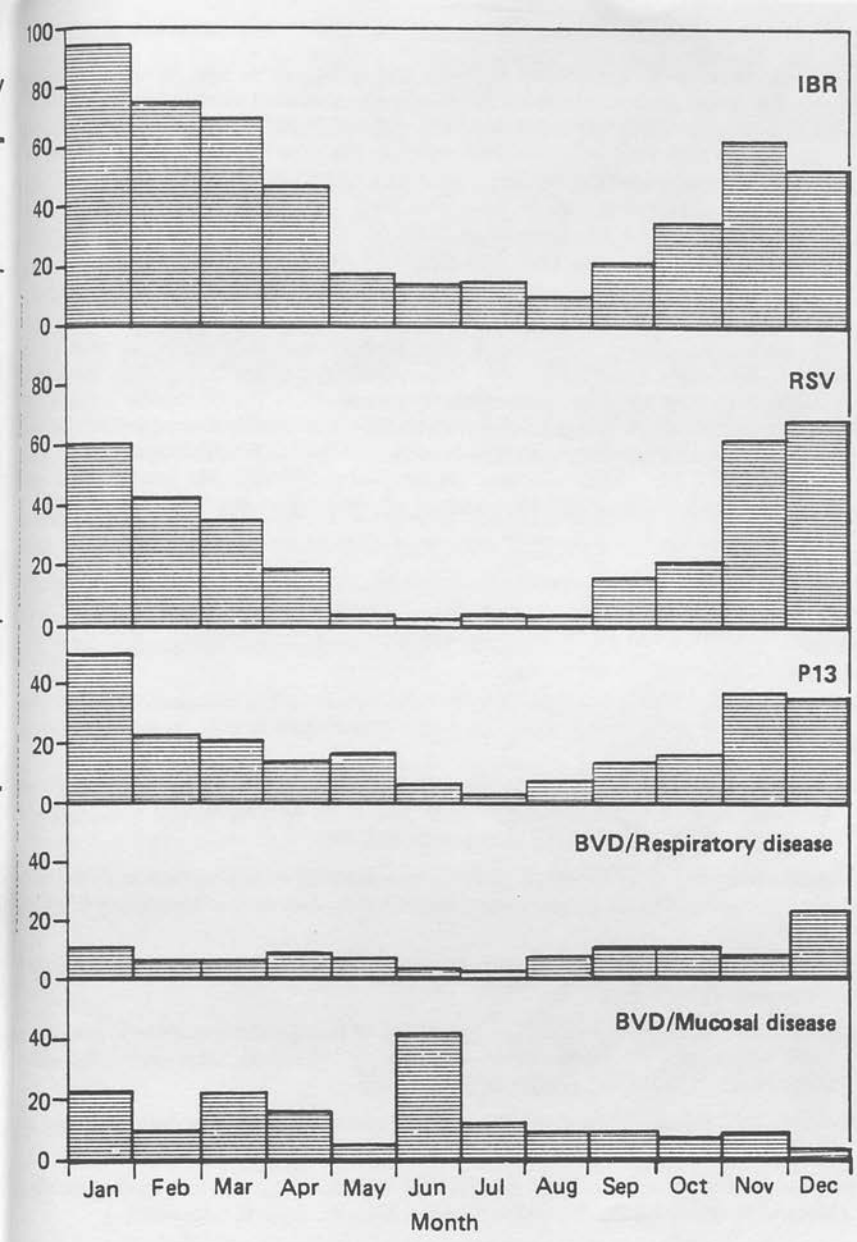
	Disease outbreaks		Live animals		PM cases	
	No. investigated	% pos.	No. tested	% pos.	No. tested	% pos.
BOVINE						
Respiratory disease						
IBR	4 347	11.8 %	6 721	8.6 %	1 935	5.4 %
RSV	3 345	9.9 %	4 670	2.1 %	2 035	14.2 %
PI3	3 278	7.1 %	4 668	3.7 %	1 899	6.3 %
BVD	1 577	6.2 %	1 656	4.6 %	830	7.7 %
Adenovirus	79	10.1 %	467	8.6 %	59	13.6 %
Mucosal disease: BVD	566	28.1 %	360	22.5 %	434	23.3 %
Abortion						
IBR	186	3.2 %	10	0	183	3.8 %
BVD	342	5.0 %	44	9.1 %	307	4.9 %
OVINE						
Respiratory disease						
RSV	49	4.1 %	22	4.5 %	26	3.8 %
PI3	159	11.9 %	63	6.3 %	121	12.4 %
Border disease	109	12.8 %	17	41.2 %	101	11.9 %

Discussion

This study has shown that a number of important viral infections of ruminants can be rapidly identified by direct IF methods for viral antigens. We have shown that the tests can be done at VICs without the need for full scale and expensive virus culture facilities. An essential component of the scheme, in the absence of commercially available reagents, is the central laboratory for the preparation of conjugates and quality control materials. Following the decentralisation of the routine testing it is also important to ensure that a proportion of samples continue to be referred to the central laboratory in order to maintain the expertise and interest of the staff there.

A particularly noteworthy feature of the work has been the low diagnosis rate for RSV in live animals, compared with a high rate at post-mortem. RSV is highly pneumotropic and the results suggest a need for wider application of lung lavage techniques for diagnosis in the live animal (Kimman and others, 1986). Adenovirus conjugates only became available latterly and the significance of the virus, and the applicability of IF to its detection, remain to be evaluated. Abortion investigations were generally unrewarding.

Preliminary studies were carried out at CVL before the main work began using indirect fluorescent labelling for viral antigen detection in bovine respiratory tissue. These gave disappointing results, with high background levels of non-specific fluorescence. Similar



igure 1. Seasonal trends in viral infections diagnosed by direct immunofluorescence at Veterinary Investigation Centres in England and Wales 1985–86.

problems have been reported by McNulty and Allan (1984) who thought them to be partly due to the quality of anti-species conjugates available commercially. There were also difficulties in using an anti-bovine conjugate on bovine tissues because most of the hyperimmune anti-viral sera available were prepared in calves. Indirect IF has been successfully used in medical virology (Gardner and McQuillin, 1980) and in veterinary diagnosis of IBR using rabbit sera (Nettleton and others, 1983). Because it is economical of viral specific antisera, indirect IF limits the requirement for a range of conjugates, but it does require a high degree of quality control checking.

Alternative techniques for antigen detection are available, notably enzyme immunoassays (Nettleton and others, 1982; Edwards and others, 1983; Harmon and others, 1979; Chao and others, 1979) which have proved especially useful in enteric virus diagnosis (Reynolds and others, 1984). Where cellular material is available, however, as in respiratory tract samples, immunofluorescence offers the particular advantage of enabling microscopical interpretation of the nature and distribution of the fluorescence and thus it enhances confidence in the results. The World Health Organization (1981) recommended that, whilst enzyme immunoassays should be evaluated further, immunofluorescence was still the method of choice for (human) respiratory virus detection.

Acknowledgements

We are very grateful to many colleagues in the Veterinary Investigation Service for their willing co-operation in this scheme.

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Establishment of a statistical base for use of ELISA in diagnostic serology for infectious bovine rhinotracheitis*

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The critical statistical parameters of an enzyme-linked immunosorbent assay were determined to enable quantitation of antibody responses in cattle affected with infectious bovine rhinotracheitis. A system of controlling well-to-well variations in optical density reading across a microtitre plate was evolved and dose–response assays were carried out to determine the dilution of serum which gave the greatest discrimination between acute and convalescent sera from an infected animal. Use of a standard serum was studied in further assays. An increase in optical density value of 0·15 was set as a diagnostic criterion for a significantly rising antibody response. This compared well with the conventional criterion of a fourfold rise in virus neutralizing antibody titre.

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) for the detection of IgG antibodies against bovid herpesvirus I (BHV I) in cattle has been shown to correlate with the virus neutralization (VN) test, although it can be more sensitive.[1,2,3] The present paper describes investigations into some of the variables which affect the ELISA. Differences in optical density reading (OD) of wells in various positions on a micro-titre plate were first examined. This was followed by a series of experiments designed to study the log dose–response curves of standard sera known to have positive antibody titres, and to define a statistically significant rise in titre from the acute to the convalescent stage of

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disease in an individual animal. This rise in OD response could then be compared with the rise in titre found in the serum VN test. Finally, results on a large number of low antibody titre sera were examined in an attempt to determine a satisfactory positive/negative cut-off point in OD reading.

MATERIALS AND METHODS

Virus strain

Bovid herpesvirus I (Oxford strain) was cultured in either primary calf-kidney or MDBK cell monolayers (shown by immunofluorescence to be free of bovine virus diarrhoea virus). When the viral cytopathic effect was advanced to the point of complete cell detachment the cells were pelleted at 1000 g and washed with phosphate buffered saline (PBS). The cell pellet was then suspended in a minimal volume of 0·5% Nonidet P40 (BDH) for one hour at 4°C. The cell debris was removed by centrifugation and the supernatant stored in small aliquots at −70°C as the BHV I antigen. Control antigen was prepared by scraping non-infected cells from the culture vessel and processing them in parallel with the viral antigen.

ELISA

Flexible immunoassay microtitre plates (Falcon 3912, Becton Dickinson) were coated in alternate rows with virus and control antigens, 100 μl per well, at an appropriate dilution (predetermined by chequerboard titration) in 50 mM carbonate/bicarbonate buffer, pH 9·6 , overnight at 4°C. The antigens were removed from the wells by decanting or aspiration, and, after washing in PBS or PBS with 0·05% Tween 20 (PBST), were dried at 37°C and stored in sealed plastic bags at −20°C.

In the assay, sera and conjugate were diluted in 0·5 M sodium chloride buffered to pH 7·2 with 0·01 M phosphate, 0·05% Tween 20, 1 mM EDTA and 0·5% ovalbumen. This buffer was derived from that of Houwers *et al.*[4] and has been found to be a more satisfactory diluent than conventional 0·15 M NaCl with 0·01 M phosphate and 0·05% Tween 20.

Serum dilutions were incubated in the plates (50 μl/well) for two hours at room temperature in a humid chamber. The plates were then washed five times with PBST.

The conjugate was anti-bovine IgG prepared in rabbits and conjugated to horseradish peroxidase by the periodate method.[5] This was used at a predetermined dilution of 1/1000 (50 μl per well) and was incubated for one hour at room temperature in the humid chamber. After five washes in PBST, orthophenylene diamine (Sigma)[6] was added, 100 μl per well. The reaction was stopped after 15 min incubation at room temperature in the dark by the addition of 25 μl per well of 2·5 M sulphuric acid. The OD was read at 492 nm wavelength in a multichannel recorder (Titertek Multiskan, Flow Laboratories).

Virus neutralizing antibody was measured as described by Frerichs *et al.*[7] The paired sera used for these experiments were from natural outbreaks of bovine respiratory disease and had been sent to the laboratory for diagnostic testing. 'Standard' sera were collected from calves immunized at the laboratory.

Experiment design and statistical analysis

Experiment 1. Two replicate plates coated with virus antigen in all wells were filled with the same dilution of positive serum (1/100) in all wells in order to study the effect

of position on the plate on OD readings. Two-way analyses of variance were carried out and results were expressed as standardized differences for rows and columns, that is, (Row mean − overall mean)/standard error for row mean, and (Column mean − overall mean)/standard error for column mean .

Experiment 2. Six pairs of acute and convalescent sera were each tested over a range of twofold dilutions from 1/50 to 1/6400. In this and all subsequent experiments each serum dilution was tested in a block pattern thus:

T1, T2
C1, C2

where T1 and T2 represent duplicates of the virus positive antigen and Cl and C2 the control antigen. Two within-block corrected OD measurements (T1–C1) and (T2–C2) were used as the responses. In this experiment the 12 sera were tested, three on a plate, allocated to columns 1–4, 5–8 and 9–12, thus filling the 24 'blocks' on the 96-well plate. The log dose–response lines were fitted over the linear portion of the curve for each serum and estimates of the response at a dilution of 1/100 were calculated in order that convalescent (B) sera could be compared with acute (A) sera.

Experiment 3. The same sera which were used in Experiment 2, that is, six pairs of acute and convalescent sera were tested, but only at a single dilution level, 1/100. Four replicate blocks of each serum, six on a plate, were tested, the blocks being randomly allocated to the 24 positions. Duplicate plates of each set were prepared.

Analysis of variance techniques were used to evaluate the results, and to compare between-block variability with that found between duplicate responses within blocks. The minimum difference in response necessary to demonstrate a significant rise in titre from the acute to the convalescent stage of the disease was estimated.

Experiment 4. Reproducibility was studied further by testing three sera, one known to have a moderately strong positive, one a weak positive and the third a negative antibody titre. Twenty-four separate dilutions of each serum were tested, each serum occupying a plate, again using the block system. Two plates were used on each of four days for each serum, making a total of 24 plates in all.

Experiment 5. The results of tests on 105 pairs of acute and convalescent sera were studied using a 1/100 dilution. The rise in OD response found in Experiment 4 to be statistically significant was taken as an indication of a positive increase in titre and this was compared with the change in titre in the VN test carried out on the same sera.

Experiment 6. Sera submitted for screening purposes (I.e. determination if negative or positive) were examined. Of these, 652 were from cattle herds known to have been free of infectious bovine rhinotracheitis (IBR) for many years and therefore true negatives, 88 were VN positives,a nd 181 were selected as 'weak positives' having given low titre reactions in the VN test. The first groups were tested at 1/100 and the weak positives at 1/100 and 1/50. Of the latter group 119 were retested on a later occasion.

Throughout these trials occasional aberrant readings occurred. Where these occurred in a 'block' of four wells, an estimate of the correct OD value (T–C) was made, corresponding to the within-block duplicate.

RESULTS

Experiment 1

Table 1 shows the standardized differences for rows and columns for the two plates tested. Values between −2 and +2 could be due to random variation, but more extreme values are likely to reflect genuine differences. These tended to occur in the two pairs of end columns of a plate, indicating an 'edge effect', but this was less marked in the top and bottom rows. Low and high values tended to occur in adjacent rows and columns, giving a clustering effect. Differences between columns are more highly significant than those between rows.

TABLE 1. Identical dilutions in 96 wells on an 8 × 12 microtitre plate. Standardized row and column effects for two replicate plates

Plate 1				Plate 2			
	Rows		Columns		Rows		Columns
A	1·9	1	−6·8	A	3·2	1	−0·5
B	2·5	2	−3·3	B	0·9	2	−0·6
C	0·3	3	2·7	C	0·1	3	1·8
D	−2·2	4	2·7	D	−1·5	4	2·0
E	−3·0	5	2·0	E	−1·3	5	2·1
F	0·2	6	2·4	F	−2·0	6	0·9
G	0·5	7	2·3	G	0·3	7	−0·4
H	−0·2*	8	0	H	0·2	8	2·0
		9	1·0			9	1·2
		10	1·1			10	0·9
		11	1·3			11	−2·6
		12	−5·5			12	−6·8
	$P < 0{\cdot}01$		$P < 0{\cdot}001$		$P < 0{\cdot}05$		$P < 0{\cdot}001$

* Replicate 1 Row H, Column 1 was an estimated value since no colour developed in this well.

Experiment 2

The log dose–response curves of ten out of the 12 sera were approximately linear over the dilution range 1/50 to 1/3200 or 1/50 to 1/6400 (see Table 2 and Fig. 1). All slopes were highly significant, and with the exception of the pair 3A and 3B the slope of the convalescent serum was considerably higher than that of the acute serum.

Responses at various dilution levels were estimated from the regression lines in an attempt to determine the most clear-cut distinction between acute and convalescent sera. A dilution of 1/100 was found to give the optimum range of readings, and at this level the difference in mean OD reading for convalescent–acute gave a better differentiation than the ratio convalescent/acute, especially with a low-level acute serum such as 5A (Table 2). The average difference, omitting 3A and 3B, was 0·70.

Experiment 3

Mean corrected OD readings for each of the 12 sera tested are given in Table 3. Mean differences between acute and convalescent sera were all very highly significant

TABLE 2. Regression of corrected mean optical density reading on log dilution for six pairs of sera

Plate	Serum	Reciprocal dilution range*	Gradient ± SE†	Response at 1/100 dilution		
				Estimate	B/A‡	B − A
	1A	50–3200	0·125 ± 0·023	0·17	4·8	0·64
1	1B	50–3200	0·466 ± 0·024	0·81		
	2A	50–3200	0·089 ± 0·016	0·11	8·6	0·84
	2B	50–6400	0·524 ± 0·033	0·95		
2	3A	50–3200	0·454 ± 0·025	0·75	1·1	0·06
	3B	50–6400	0·478 ± 0·040	0·81		
	4A§	50–6400	0·170 ± 0·009	0·29	2·6	0·46
3	4B	50–6400	0·380 ± 0·026	0·75		
	5A	50–400	0·060 ± 0·018	0·03	18·3	0·52
	5B	50–6400	0·328 ± 0·029	0·55		
4	6A	50–3200	0·095 ± 0·013	0·15	7·9	1·04
	6B	200–6400	0·626 ± 0·012	1·19		

* Reciprocal dilution range over which log dose–response curve was linear.
† Gradient ± SE: Regression coefficient and its standard error.
‡ Serum A = acute, B = convalescent.
§ Serum 4A: Responses at 1/200 and 1/1600 omitted due to significant deviations from linearity.

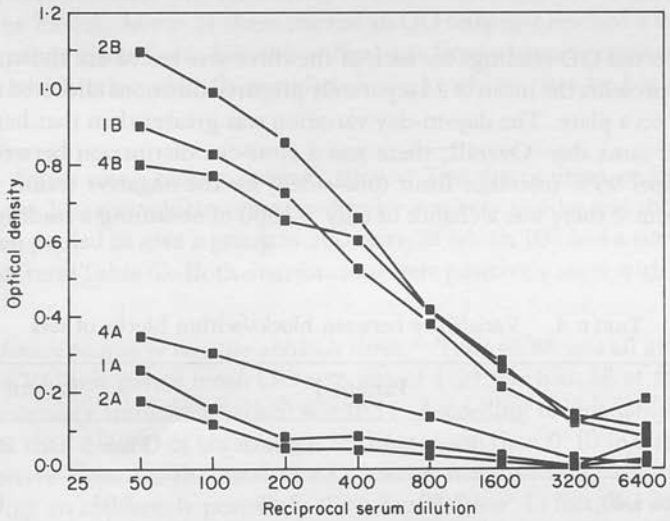

Fig. 1. The log dose–response curves for three pairs of acute (A) and convalescent (B) sera assayed in Experiment 2.

TABLE 3. Mean optical density readings of six pairs of sera tested at a dilution of 1/100

Pattern	Plate	Mean corrected O.D. values for 4 blocks of 2 replicates						
		Serum*						
		1A	1B	2A	2B	3A	3B	LSD†
1	1	0·136	0·792	0·106	0·850	0·703	0·771	0·096
	2	0·183	0·780	0·099	0·832	0·671	0·746	0·091
		Serum						
		4A	4B	5A	5B	6A	6B	
2	3	0·299	0·844	0·049	0·589	0·174	0·987	0·114
	4	0·350	0·956	0·040	0·721	0·134	1·090	0·152

* A = Acute, B = convalescent.
† LSD = Least difference between two means necessary to show significance at the 5% level.

($P < 0{\cdot}001$) with the exception once more of the low level pair 3A and 3B which did not differ significantly in any test. The difference between mean corrected responses of four replicate blocks of readings for each serum required to show a difference which is significant at the 5% level averaged at 0·113. If only a single block of duplicates per serum was used, the difference necessary for significance would be 0·226.

The variance (F) ratios of the difference between blocks of the same serum in different areas of the plate to that between duplicates within blocks was significant for each of the four plates tested (Table 4).

Experiment 4

Mean corrected OD readings for each of the three sera tested are shown in Table 5. Each value represents the mean of 24 separately prepared dilutions allocated to the blocks of four wells on a plate. The day-to-day variation was greater than that between plates tested on the same day. Overall, there was a clear-cut distinction between the three sera. The upper 99% tolerance limit (one-sided) for the negative serum 1 was 0·06, while for serum 2 there was a chance of only 1/1000 of obtaining a reading lower than 0·16.

TABLE 4. Variability between blocks/within blocks of sera

	Pattern 1		Pattern 2	
	Plate 1	Plate 2	Plate 3	Plate 4
F ratio (18, 24 DF)	7·80	2·28	2·03	13·43
Significance (P)	< 0·001	< 0·05	0·05	< 0·001

TABLE 5. Mean optical density (OD) readings of 24 separate dilutions per plate for each of three sera

	Serum 1 Negative		Serum 2 Weak positive		Serum 3 Medium positive	
Day	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
1	0·027	0·050***	0·254	0·216***	0·685	0·698
2	0·027	0·020	0·292	0262**	0·672	0·596**
3	0·010	0·013	0·275	0·333***	0·762	0·746
4	0·016	0·012	0·238	0·247	0·739	0·723
SEM of 24 values	0·0032		0·0066		0·0112	
Overall mean OD	0·022		0·265		0·703	
LSD† (5% level)	0·043		0·090		0·152	
LSD† (1% level)	0·057		0·119		0·200	
95% C.L.‡ of a single result	± 0·031		± 0·064		± 0·108	

Significant difference between plates, ** = $P < 0{\cdot}01$, *** = $P < 0{\cdot}001$.
† LSD = least difference necessary for significance between individual dilutions within a plate.
‡ C.L. = Confidence limit.

Experiment 5

From the findings in Experiment 4 it would appear that a change in corrected OD reading of 0·15 can be accepted as statistically significant (Table 5), and if the rise from the acute to the convalescent stage in a pair of sera reached this level in the present experiment it was interpretated as positive. The criterion for a significant rise in seroneutralization (VN) titre ($\log_2$) was taken as 2·0 (i.e. a fourfold rise in titre), and on this basis 87 of the 105 sera examined (83%) gave a positive result to both tests, while 16 were negative to both. The remaining two sera were negative to the VN test but positive in the ELISA. In one of these the rise in OD only just reached a value of 0·15, while in the other it was 0·46, but this animal was from a group experiencing an IBR outbreak in which other animals sampled showed a rising titre by both tests.

Experiment 6

Sera with known weak positive antibody titres. The distribution of corrected OD readings on the 119 sera which were tested twice was very similar and the results have therefore been pooled to give a group of 300 tests, of which 107 had a titre of less than 1/4 in the VN test (Table 6). Both distributions were positively skew with considerable overlap.

Sera with known positive or negative antibody titres. Tests on 88 sera all giving positive results in the VN test gave a mean OD response of 1·21, with an SE of ± 0·045. The lower 95% tolerance limit (one-tailed) was 0·51. According to this sample, there is a chance of less than 1/1000 of obtaining a reading of less than 0·10 in a sample with a strongly positive titre. Of the total of 652 known negative sera, 157 had zero OD readings giving an apparently positively skew distribution. In fact, the distribution is probably normal, with many of the zero readings having a numerically negative value. No readings exceeded 0·10.

TABLE 6. Distribution of corrected optical density (OD) readings of sera assessed as positive or negative according to the virus neutralization test

300 weak positive sera			740 sera			
			652 Known negatives		88 VN positives	
OD	VN < 4	VN > 4	OD	VN (−ve)	OD	VN (+ve)
0	31	6	0·00	157	0·10–0·19	5
0·01–0.04	35	9	0·01	68	0·20–0·29	2
0·05–0·09	24	7	0·02	75	0·30–0·39	0
0·10–0·14	10	26	0·03	74	0·40–0·49	2
0·15–0·19	3	33	0·04	64	0·50–0·59	1
0·20–0·24	2	21	0·05	67	0·60–0·69	2
0·25–0·29	0	17	0·06	58	0·70–0·79	4
0·30–0·39	2	33	0·07	53	0·80–0·89	1
0·40–0·49		18	0·08	35	0·90–0·99	2
0·50–0·59		11	0·09	1	1·00–1·09	3
0·60–0·69		4			1·10–1·19	9
0·70–0·79		1			1·20–1·29	7
0·80–0·89		3			1·30–1·39	15
0·90–0·99		1			1·50–1·59	12
1·00–1·09		0			1·60–1·69	7
1·10–1·19		1			1·70–1·79	2
1·20–1·29		0				
1·30–1·39		2				
Total	107	193		652		88

DISCUSSION

It has been shown by a number of workers that variations in optical density readings occur between wells in different positions on a micro-titre plate. Kricka *et al.*[8] examined plates of various types using four separate assay procedures. They demonstrated that higher absorbance values frequently occurred round the edges of the plate than in the centre, possibly due to a birefringence effect, and that high, medium and low readings tended to occur in clusters in adjacent rows and columns. In Experiment 1 of the present trial, and in other studies at this laboratory, similar effects have been observed, and it was found that differences between columns were almost always greater than those between rows. The pattern is not necessarily the same when two plates are tested consecutively with the same dilutions, nor is the overall mean level of absorbance the same on both plates. There is a tendency for low (or high) row and column means to be grouped together giving a clustering effect. The greater range in the standardized column effects indicates that these differ more significantly than do the row effects.

That the block system considerably reduces variability is shown in the present experiments where differences between blocks in different areas of a plate are consistently, and generally statistically significantly higher than intra-block variation. When designing an experiment it is usually advisable to assign the sera under test to

these 24 blocks of four wells each in a systematic pattern (although this can still be 'random') taking into account the likelihood that higher absorbance will probably occur round the edges of the plate particularly in the first two and last two columns.

In designing the layout of sera on the plate it is also important to consider technical aspects of the addition of the solutions and to follow a straightforward pattern which will avoid errors of technique.

Despite some deviations from linearity it has been shown that it is possible to fit regression lines to sera over a range spanning more than 100-fold difference in dilution. This is of use in assessing the potency of test sera in relation to a standard of known potency. Since day-to-day variations between assays are almost always greater than those between plates tested on the same day, the standard should be included at three or more dilution levels at least once in each day's assay. This will serve as a working quality control check. Care should of course be taken when expressing OD readings in terms of the standard where these fall in the area of low slope at either end of the sigmoidal curve.

The greater the activity of the serum the steeper will be the slope of the log dose–response curve, and significant deviations from parallelism of the curve may occur, as pointed out by de Savigny & Voller.[9] It has been shown[10–12] that ELISA results, whether expressed as OD values or as end-point titres, are affected by both the concentration of antibodies in the serum and their affinity, and that the effects of affinity vary at different serum dilutions in the assay. Tests at a single serum dilution tend to measure high affinity antibodies whereas end-point titres relate to a larger range of affinities. These factors may account for the differences in slope of the dilution curves for different sera in the present study. Despite the lack of parallelism, the use of a single test dilution to compare sera from the same animal can be justified in that the change in slope will tend to enhance differences between acute and convalescent sera and thus improve the test's value for quantitative diagnositc assay.

The log dose–response curve was used to assess the optimum dilution at which field sera should be tested in order to give optical density readings the majority of which, after correction for the corresponding control values, will fall within the range 0·10–1·90. In our experiments this dilution was found to be 1/100, since this gave the greatest differentiation between convalescent and acute sera. A dilution of 1/200 would also have given a satisfactory range of readings, but the difference convalescent − acute was less consistent than the ratio convalescent/acute. In practical terms a ratio is less satisfactory than a difference since it can be seriously misleading if the acute serum (the denominator) has a very low value. Ratios are, however, widely used to determine a positive response to the presence of antibodies and de Savigny & Voller[9] have put forward a number of other approaches to estimate relative 'antibody activity'. They concluded that no system was ideal.

When low titre sera were being tested as in Experiment 6 it was found that a dilution of 1/50 was more satisfactory than one of 1/100, and this is recommended for use in a screening test where a high proportion of negative values is expected.

When the paired sera were tested by both the ELISA and the VN test, there were only two discrepancies out of a total of 105 pairs of samples. Of these, one had a rise in OD which was just significant but it had a static VN titre. This may possibly be accounted for by a test error in the VN (which was not repeated) or by the fact that the two tests do not necessarily measure the same antibodies.

The occasional 'aberrant' OD values recorded in individual wells are thought to be

associated with technical errors in performance of the test. This view was reinforced by the observation that the frequency of such aberrant values decreased as operators became more familiar with the technical procedures. In routine diagnostic testing they will be detected by a discrepancy between the replicates within a block and such samples would be retested.

Actual corrected OD readings for the 72 sera can be defined as positive or negative by reference to the VN titre. This confirms earlier findings, namely, that the distribution of positive readings is very wide with occasional zero or very low values, and also that there is a tendency to positive skewness particularly in the distribution of negative readings. This fact was also noted by de Savigny & Voller,[9] who suggested that non-parametric methods should be used in defining an upper limit for negative sera. Some of the high reading 'negative' sera may be weak positives detected only by the ELISA with its greater sensitivity as described by Bolton *et al.*[1] Inevitably there is an overlap between the distribution of OD values for negative and positive sera and some sera will remain indeterminate.

However, from the results in the final experiment it appears that, while sera with weak positive antibody titres can give very low OD readings, for a true negative serum there is a probability of less than 1/500 that the readings will exceed 0·10. In Experiment 6 there was one such value, which was omitted from the calculations since it was an outlier.

Although the VN test is widely accepted as the reference method for assessment of BHVI antibodies, it is also recognised that it suffers from interference by non-antibody neutralizing factors in some sera.[13,14] It is suggested that such factors may account for some of the discrepant readings, particularly where weak positive VN sera were negative by ELISA. In such cases the ELISA may be the more accurate test.

Although some authors have recommended highly purified antigens for ELISA[1,3] others have achieved satisfactory results with a crude antigen.[15,16] The unpurified antigen used in the present experiments is very simply prepared with basic laboratory facilities and has performed well with experimental and field sera.

Non-specific reactivity, detected by high OD with the control antigen, is a problem with some field sera which have been handled or stored. The block testing system used here, incorporating control antigen, will detect such sera and will still allow assessment of virus specific antibodies except in the occasional samples with very high control readings.

The use of ELISA in quantitative diagnostic serology for IBR has been established, and a critical level determined for significant rises in OD (0·15) between acute and convalescent sera tested simultaneously at a single dilution of 1/100. For screening of sera in conjunction with IBR control schemes, testing prior to export, etc., the maximum sensitivity was achieved at 1/50 serum dilution, with a calculated OD of 0·10 as the positive/negative cut-off point.

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